Revisiting chromosomal diversity in *Otomys irroratus*: meiotic behaviour and mitochondrial divergence

By

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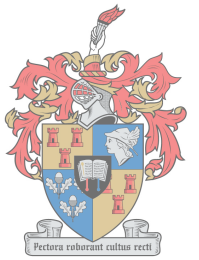

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Abstract

In this study, immunofluorescence allowed the evaluation of meiotic chromosomal behaviour of the vlei rat, *Otomys irroratus*, a species documented for its karyotypic variability. This species was recently reported to form a species complex, as high sequence divergence values of the mtDNA cyt *b* gene, revealed a cryptic sister species, *O.auratus*. Pericentric inversions, tandem- and Robertsonian fusions are found in *O. irroratus* s.l karyotypes, however these classes of rearrangements are often negatively heterotic. Immunofluorescence was used to provide insight into the persistence of these chromosomal rearrangements believed to be deleterious to the carrier. The aim was to uncover the mechanisms which override the deleterious consequences of chromosome rearrangements, and gain a better understanding of the chromosomal evolution in this species. Firstly, specimens were G- banded to identify chromosomal rearrangements. Secondly, specimens were immunostained to assess the influences of chromosomal rearrangements on meiotic configurations. Lastly specimens were sequenced for the mtDNA cyt *b* and COI genes to determine to which extent chromosomal rearrangements correlate with mtDNA clades.

The cytogenetic screening revealed two general cytotypes. The first has karyotypes which consists of 4 – 9 bi-armed chromosomes (2n= 28 - 30/NA= 44 – 50) and an abundance of rearrangements that appears as either pericentric inversions or centromere shifts on OIR 1, 4, 6,7 and 10. From these, the rearrangement on OIR 7 was described for the first time. The immunofluorescence trials with the SYCP3 antibody, targeting the proteins of the lateral elements of the synaptonemal complex revealed no inversion loops, even in specimens indicated to carry inversions by G-banding data. Rearrangements therefore did not appear to hamper pairing of chromosomes during meiosis. In addition, one specimen was indicated to have two centromeric signals per chromosome, by the CENPC antibody, which targets

centromeric proteins. This suggests that a centromeric shift took place recently with the second centromere likely being an evolutionary new centromere. The immunostaining data hence casts doubt onto pericentric inversions, and rather suggests centromeric shifts. Also these rearrangements does not seem to affect reproduction given the data gathered here. The second cytogenetic group (2n= 24, 30/FA= 32 - 37) has karyotypes with acrocentric chromosomes, while the fusion chromosome involving OIR 7, 8 and 12, as well as Rb fusion 1/6 are also present in this group. These cytotypes differ in chromosomal arms (NA), 2n, rearrangements present, and localities where they have been found and is consistent with the two main chromosomal blueprints described for *O. irroratus* s.l.

The mtDNA sequence data revealed two large clades with 5.6% divergence (uncorrected p-distances) that corresponds to the two cytogenetic groups. These groups have the same geographical distribution as the two main cytotypes, i.e. clade 1 encompassing the Western and Eastern Cape, whereas clade 2 stretches north-eastwards from the Eastern Cape to Kwazulu-Natal. These groupings were retrieved with both the cyt *b* and the COI genes, indicating that the COI may be a useful marker for phylogeographies of small mammals. The sequence divergence values were compared among specimens with and without inversions and revealed that specimens without inversions are equally divergent from specimens with one or many inversions. In effect the data suggests that inversions are not a strong driver for mtDNA divergence in the species complex. The sequence and immunofluorescence data therefore substantiate the idea that these rearrangements exist as neutral polymorphisms.

Opsomming

In hierdie studie, was immunofluoressensie gebruik om die meiotiese chromosoom gedrag van die vlei rot, *Otomys irroratus*, ‘n spesie bekend vir sy chromosoom variasie, te ondersoek. Hierdie spesie was onlangs raporteer as ‘n spesies kompleks, aangesien hoë mtDNA skeidings syfers ‘n kriptiesie suster spesie, *O. auratus* openbaar het. Perisentromeriese inversies, tandem en Robertsoniese fusies kom voor in die kariotipes. Hierdie klasse van chromosoom afwykings word gekoppel aan negatiewe heterose, maar ongeag is hulle, veral perikentriese inversies, volop in die kariotipes van *O. irroratus* s.l. Immunofluoressensie kon hier die teenwoordigheid van hierdie afwykings help verstaan, en nuwe insigte van die chromosomale evolusie van die spesie skep. Die doel was om te ondersoek of daar meganismes in plek is, wat die nadelige gevolge van hierdie chromosoom afwykings verhoed. Monsters was eerstens analiseer met G-bandbepaling om strukturele chromosoom herrangskikkings te identifiseer, tweedens het immunofluoressensie die invloed van herrangskikkings op meiotiese chromosome bepaal. Laastens, het mitochondriale volgordebepaling van die sitochroom *b* en sitochroom oksidasie I gene, ondersoek of chromosoom afwykings met mitochondriale groepe korreleer.

Die sitogenetiese analise het twee hoof chromosoom groepe opgelewer. Die eerste groep se kariotipes (2n= 28 - 30/Nfa= 44 – 50) het heterokromatiese kort arms op 4 – 9 chromosoom pare, en kan gekenmerk word deur chromosoom herrangskikkings wat as perisentromeriese inversies op outosome OIR1, 4, 6, 7 en 10 voorkom. Die inversie op OIR 7 was hier vir die eerste keer beskryf. Die immunofluoressensie experimente met die SYCP3 antigeen, wat die laterale elemente van die synaptomale kompleks uitwys, het geen inversie lusse getoon nie, ‘n onmisbare kenmerk van inversies. Chromosoom afwykings het nie meiotiese chromosoom paring beinvloed nie. Een monster wat met die CENPC antigeen

bestudeer was, het twee sentromeer seine per chromosoom in een paar opgelewer. Dit dui waarskynlik op ‘n nuwe evolusionêre sentromeer wat besig is om te vorm voordat die ou een heeltemal kon verval. Die resultate skep twyfel oor die identiteit van die chromosoom afwykings wat voorheen as perikentriese inversies aangeteken is, aangesien dit beter as sentromeer verskuiwings vertoon. Die tweede chromosoom groep (2n= 24, 30/Nfa= 32 - 37) het geen inversies en bestaan uit kariotipes met akrocentriese chromosome. ‘n Tandem fusie van OIR7, 8, en 12 asook die Robertsoniese fusie van OIR 1/6 kom voor slegs in die groep. Die twee groepe verskil dus in chromosoom vorm (met betrekking tot hoeveelhied fundementele chromosoom arms-Nfa), diploïede getal (2n), afwykings teenwoordig asook geografiese verspreiding en is konsekwent met die twee hoof chromosoom groepe wat voorheen vir die spesies kompleks beskryf is.

Die mitokondriale data het twee klades wat met 5.6% (onkorregeerde p- afstande) verskil opgelewer, en stem ooreen met die twee chromosoom groepe. Hierdie klades het dieselfde geografiese verspreiding as die chromosoom groepe naamlik clade 1 wat die Wes- en Oos-Kaap insluit, terwyl clade 2 verder Noord-Oos van die Oos-Kaap strek tot Kwazulu-Natal en Zimbabwe. Beide mitokondriale merkers het dieselfde groepe oopgelewer, wat dui dat die sitokroom oksidasie I merker ook nuttig in filogeografiese studies van klein soogdiere kan wees. Die mitokondriale volgorde verskille was vergelyk tussen monsters wat inversies besit en die wat nie, en daar was gevind dat monsters wat nie inversies het nie, ewe ver geskei is van monsters wat een of meer inversies besit. Die resultate dui daarop dat inversies nie ‘n sterk dryfkrag vir mitokondriale skeiding in die spesies kompleks is nie. Die resulte hier ondersteun die idee dat hierdie chromosoom herrangskikkings as neutrale polimorfismes ontstaan.

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Table of Contents

List of tables

List of figures

Acronyms and abbreviations

°C - Degrees Celsius

2n – Diploid number

$Ba(OH)_2$ – Barium hydroxide

BAC – Bacterial Artificial Chromosome

CENPC – Centromeric Protein C

CO – Cross Overs

CO_2 – Carbon Dioxide

COI – Cytochrome Oxidase 1 subunit

Cy3 – Cyanine 3

cyt *b* – Cytochrome b subunit

DAPI - 4',6 diamidino-2-phenylindole

dHJ - Double Holliday Junction

DMC1 - Disrupted Meiotic cDNA 1

DMEM - Dulbecco's Modified Eagle Medium

DSB's – Double Strand Breaks

FCS - Foetal Calf Serum

FITC - Fluorescein Isothiocianate

H3K9me3 - Histone 3 trimethylated at lysine 9

IF- ImmunoFluorescence

KCl – Potassium Chloride

KH_2PO_4 – Potassium dihydrogen phosphate

MLH1 - MuTL Homolog 1

MLH3 - MuTL Homolog 3

MSH4 - MutS Homologs 4

MSH5 - MutS Homologs 5

mtDNA - Mitochondrial DNA

OIR – *Otomys irroratus* (chromosome)

PBS - Phosphate-Buffered Saline

Rad51 - Radiation sensitive 51

Rb – Robertsonian (fusion)

REC8 - Recombinant 8

SC – Synaptonemal Complex

SMC1 - Structural Maintenance of Chromosomes 1

SSC - Saline Sodium Citrate

SYCP3 – Synaptonemal Complex Protein 3

MYA – Million Years Ago

Chapter 1: Introduction

1.1 Preamble:

The vlei rats *Otomys irroratus* (Brants, 1827) and *Otomys auratus* Wroughton, 1906, can be found respectively, in the mesic habitats of the cape fynbos and grasslands of sub-saharan Africa (Monadjem *et al.* 2015). Originally viewed as one species, *Otomys irroratus* s.l. is remarkably conserved in phenotype, but displays extreme chromosomal variation, with diploid chromosome numbers ranging from 2n= 23 -- 33 (Contrafatto *et al.* 1992a, b, Robinson and Elder 1987, Rambau *et al.* 2001). This variation is due to Robertsonian fusions (some of which occur as floating polymorphisms), and a compound tandem fusion resulting in two basic cytotypes 2n= 24 and 2n= 28 (Rambau *et al.* 2001). Mate choice experiments between vlei rats from two distinct and geographically isolated populations in the Kwazulu-Natal province, one with and without this tandem fusion, indicate that rodents prefer homotype-stimulus (Pillay *et al.* 1992), suggesting premating reproductive isolation between different vlei rat populations. The apparent divergence between these two cytotype blue prints is supported by mtDNA sequence- and morphometric data (Taylor *et al.* 2009a; Engelbrecht *et al.* 2011a). Against this background; *O. irroratus* s.l. provides the ideal opportunity to test the theories of chromosomal speciation: Recombination suppression and Hybrid dysfunction (Faria and Navarro 2010, Brown and O'Neil 2010), to uncover the mechanisms that allow the persistence of chromosomal rearrangements, which are normally thought to be negatively heterotic.

In this study I used standard cytogenetic techniques to karyotype and analyse new populations of *O. irroratus* s.l. in the context of previous published data. I discuss the general distribution patterns of chromosome rearrangements and explain how it may drive evolution in

the species. A combination of mtDNA and immunostaining data provides insights into the role of chromosomal reorganizations in shaping the evolutionary patterns seen in *Otomys*.

1.2 Evolutionary history in the Otomyinae

The Otomyinae is a subfamily of the Murinae, which forms part of the Muroidae superfamily. This group is often given tribal status (Otomyini - Michaux *et al.* 2001, Jansa and Weksler 2004), although some molecular studies classify it as a subfamily (Musser and Carleton 2005). Systematic undertakings making use of the full mitochondrial dataset of the genes 12S + 16S + cytochrome *b* (cyt *b*), grouped four representatives of this family basal to *Aethomys* within the Muridae. The Otomyinae are comprised of three genera, namely *Myotomys* Roberts 1929, *Otomys* and *Parotomys* Thomas 1918 which originated in South Africa approximately 11 mya (Sénégas 2001, Lecompte *et al.* 2008). The type genus *Otomys* originated roughly five mya (Avery 1991, Denys 2003), with the oldest fossil dating 3 – 3.7 mya (Sénégas and Avery 1998, Taylor *et al.* 2004a, 2009b). Throughout the evolutionary history of this genus, species have remained remarkably conserved in phenotype, often making identification in the field a challenging task.

The Otomyinae are distinguished from other murid rodents by their heavy and deeply grooved upper incisors and laminated molar teeth (Smithers 1983). These African endemics are comprised of nine South African species known as the karoo rat and ice rat *Myotomys* Thomas, 1918 (*M. unisulcatus* F. Cuvier, 1829 and *M. sloggetti* Thomas, 1902 respectively), laminate toothed rats *Otomys* (*O. irroratus*, *O. auratus*, *O. angoniensis* Wroughton , 1906, *O. karoensis* Roberts, 1931 *O. laminatus* Thomas & Schwann, 1905) and the whistling rats, *Parotomys* Thomas, 1918 (*P. brantsii* A. Smith, 1834 and *P. littledalei* Thomas, 1918 – Monadjem *et al.* 2015). The taxonomic validity of these groupings has been questionable due to incongruences in the findings of different methods of analysis. *Otomys* and *Parotomys* can

for example be separated by the size of their auditory bullae which is smaller in *Otomys* (≤9.5 mm) than in *Parotomys* (≥10.8 mm, Meester *et al.* 1986), but not by sperm morphology (Bernard *et al.* 1991), and allozymes or craniodental characters (Taylor *et al.* 1989, Contrafatto *et al.* 1997, Taylor *et al.* 2004b). The Otomyinae can however, to an extent be distinguished by their habitat preference. *Otomys karoensis*, *M. unisulcatus* and the two *Parotomys* species are for example xeric adapted, occurring mostly along the western parts of South Africa whereas other *Otomys* species are more mesic adapted, occupying the eastern parts of South Africa. *Myotomys sloggetti* is the only Otomyine species found in cold alpine environments such as the Drakensberg and Maluti mountain ranges, hence it is often called the ice rat (Skinner and Chimimba 2005). Molecular studies (employing nuclear and mitochondrial markers), offered better resolution of the taxonomy of the Otomyinae and recognized the genus *Myotomys* where its members where previously grouped as part of *Otomys* (Musser and Carleton 2005, Lecompte *et al.* 2008). In this family *O. irroratus* is unique as displays broad scale chromosomal variation across its geographic range. The various chromosomal races / cytotypes occurring in this taxon encouraged the taxonomic re-evaluation which uncovered a cryptic lineage divergent enough to be recognized as valid species (Contrafatto *et al.* 1992a, b, Taylor *et al.* 2009a, Engelbrecht *et al.* 2011b) hence leading to the description of *r* (Monadjem *et al.* 2015).

1.3 Life history of *Otomys irroratus*

Vlei rats are primarily crepuscular although field and laboratory activity tests indicated some activity in the day and night (Skinner and Chimimba 2005). Consistent with all members of this genus, vlei rats are herbivorous eating a wide variety of plant species occurring in their habitat. These rats generally occur solitarily or in pairs and have been found to be anti-social and territorial, showing aggression to intruders of their own species (Davis 1972). They construct saucer shaped nests on rising dry ground or in clumps of grass (Smithers 1983), and

occasionally use burrows of other species (Bronner 1992, Skinner and Chimimba 2005). Vlei rats reproduce throughout the year reaching a peak during the warm wet summer months (August to early May) of high food availability (Davis and Meester 1981, Skinner and Chimimba 2005). Vlei rats are prolific breeders with litter sizes ranging from two to 12 young in one season (Smithers 1983).

1.4 Incipient speciation in *Otomys irroratus* complex

The phenotypic conservatism observed in *Otomys* makes separation of members based on morphology a difficult task. It has emerged that species within *Otomys* can be better separated on the basis of bioregion and habitat preference (Engelbrecht *et al.* 2011a, Mucina and Rutherford 2006). The taxonomy of *O. saundersiae*, now no longer considered a valid taxon, was formerly described as the sibling species to *O. irroratus*, was associated with the rocky outcrops of the fynbos biome in the Western Cape Province, under the denomination *Otomys saundersiae karoensis*. This vlei rat later described as *O. karoensis s. s.* is also smaller in size with a pallid and buffy pelage colour whereas *O. irroratus* is larger in size, have a darker brown colour and occurs on valley bottoms. In comparison to these two, *O. saundersiae* is intermediate in size, have a buffy colouration and occurs on upper slopes of mountains (Taylor *et al.* 1993; Taylor *et al.* 2009a). The range of *O. saundersiae* further extends into the summer rainfall areas of the Southern Drakensberg (Grassland biome) and the coastal regions of the Eastern-Cape (Albany thicket Biome). The type locality of the former described *O. saundersiae* was encompassed by this East coast region around Grahamstown.

Originally *O. karoensis* was regarded as a subspecies of *O. saundersiae* (Meester *et al.* 1986). Taylor *et al.* (1993) later found that the two taxa can be distinguished in the Fynbos biome of the Western Cape and the Eastern Cape north of 32° latitude (Grassland Biome > 1200 m), but not in the lower lying parts of the Eastern Cape (Albany Thicket Biome < 1200

m, including topotype of *O. saundersiae*) south of the 32° latitude. Taylor *et al.* (1993) therefore suggested including *O. saundersiae* under *O. irroratus* and elevating *O. karoensis* to species rank. Subsequent morphometric analysis however failed to group *O. saundersiae* with *O. irroratus* but rather grouped it with *O. karoensis* (Taylor *et al.* 2005). The ambiguity in the data regarding *O. saundersiae*'s position prompted Taylor *et al.* (2009a) to utilize a combined molecular, cranial morphometrics and cytogenetic approach in order to provide resolution to the position of *O. irroratus*, *O. saundersiae* and *O. karoensis* relative to each other. Both the cyt *b* as well as the karyotypic data refuted the taxonomic validity of *O. saundersiae*. Both karyotypic (bi-armed chromosomes) and cyt *b* datasets grouped *O. saundersiae* with *O. irroratus*, rather than with *O. karoensis* as in the previous study (Taylor *et al.* 2005). Only the cranial morphometric data supported the validity of *O. saundersiae* as a taxon, however the integrity of molecular data cannot be disputed. The karyotype of *O. karoensis* is significantly different from *O. irroratus*, with only four shared homologous chromosomal pairs (showing complete homology/retention of chromosomes-the remainder all being rearranged). The karyotypic data therefore supports the recognition of *O. karoensis* as a species (Taylor *et al.* 2009a).

A noteworthy finding of Taylor *et al.* (2009a) and Engelbrecht *et al.* (2011a) was the detection of at least two evolutionary species within the *O. irroratus* complex. These two species, a Fynbos and Albany thicket biome species as well as a northern Grassland biome species have diverged 1.13 million years ago and are demarcated by 6.4% cyt *b* sequence divergence. It was previously thought that these lineages only differ with regards to karyotype, but it appears that these lineages are better adapted to biome (Taylor *et al.* 2009a). The northern lineage from the Grassland biome has been renamed *O. auratus* (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a).

1.5 Chromosomal variation in *O. irroratus* s.l.

Within *O. irroratus* s.l., the diploid numbers vary between 2n= 23 and 2n= 33, and were previously divided into four cytotypes A1, A2, B and C. These groups differ due to the presence/absence of B chromosomes (supernumerary chromosomes comprised of heterochromatin) a tandem fusion (the end to end fusion of one chromosome with another) as well as the addition or deletion of heterochromatic short arms. Furthermore Robertsonian (Rb) fusions (the fusion of chromosomes at the centromere) and pericentric inversions (a rearrangement that flips the gene content around the centromere of a chromosome) exists in the karyotypes as floating polymorphisms (see Robinson and Elder 1987, Engelbrecht *et al.* 2011b). These groups have a parapatric distribution (Figure 1.1).

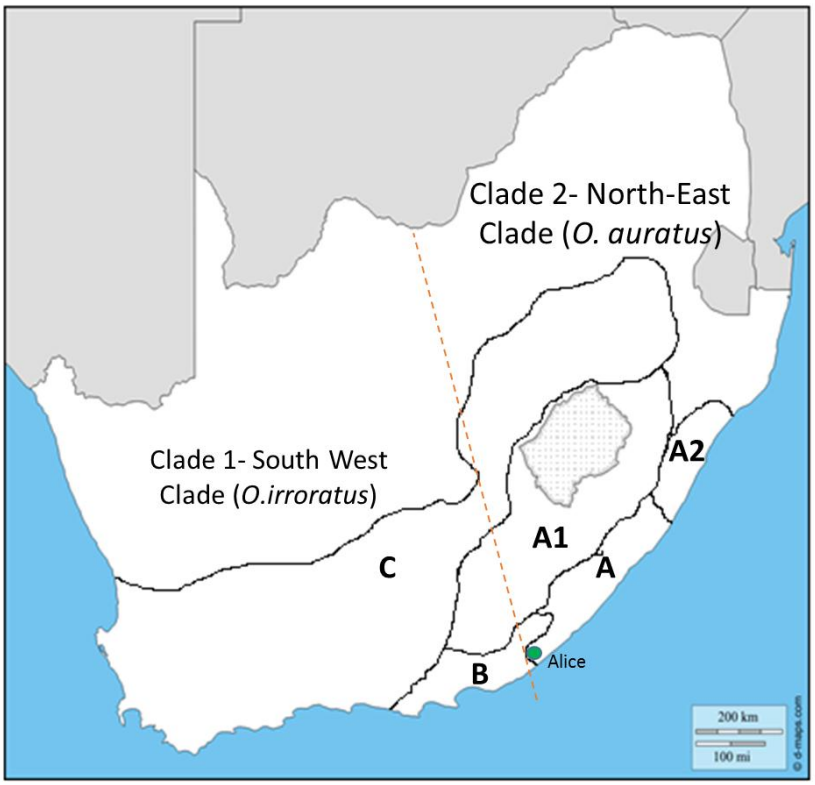


Figure 1.1. Distribution of *O. irroratus* s.l cytotypes A1, A2, B and C. The A cytotype represents the acrocentric chromosome type which lacks heterochromatin and further includes the A1 cytotype which can be found along the East coast and inland South Africa whereas A2 is restricted to the high altitudes of the Drakensberg in KZN. The B and C cytotypes has heterochromatic short arms on 6 – 8 and 4 autosomal pairs respectively. Cytotype B is found on a localized stretch in the Eastern Cape, whereas C has the widest distribution ranging from the Southwestern Cape to the North-eastern Cape inland. Dotted line roughly indicates the separation of the two lineages (clade 1 and clade 2), which are sympatric around Alice in the Eastern Cape.

Cytotype A is comprised of acrocentric chromosomes only and can be subdivided into two chromosomal races A1 and A2, which generally has a 2n= 24 and 2n= 28 respectively. These two subgroups differ due to a large compound chromosome that is present in the A1 karyotype, but absent in the A2 karyotype. This compound chromosome is the result of the fusion of *O. irroratus* chromosomes 7, 12 and 8. Using mouse chromosome paints (MMU) Engelbrecht *et al.* (2006) detected homology between OIR 7 and MMU 17+18, OIR 8 and MMU 1+15 as well as OIR 12 and MMU 12. The A1 cytotype has been recorded along the East Coast, and further inland of South Africa, in Hogsback (Eastern Cape) and in Kamberg (KwaZulu-Natal). The A2 cytotype has been documented in KwaZulu-Natal on higher altitudes of the Drakensberg highlands. Unlike the other cytotypes, A does not have any heterochromatin, and has two *de novo* autosomal pairs whose homology has not yet been established (Contrafatto *et al.* 1992b).

Cytotype B are characterised by heterochromatic short arms (p) in six to eight autosomal pairs and a 2n= 28. This cytotype can mainly be found in the Eastern Cape around Port Elizabeth (Contrafatto *et al.* 1992b). Cytotype C has heterochromatic short arms on autosomal pairs OIR1 – 4, also with a 2n= 28. This group has the widest distribution range in South Africa, stretching from the South-Western Cape, northwards into the interior and further North Eastwards to the Limpopo province (Figure 1.1). There appears to be a correlation between these chromosomal karyotypes and climate (Taylor *et al.* 1994, Engelbrecht *et al.*

2011a), which suggests that certain cytotypes may confer an adaptive advantage in the climate in which they occur. Specimens from the cold, moist, high altitude grasslands from Hogsback (A1 cytotype- with tandem fusion) for example had a significantly lower winter trap mortality (4.8%) than specimens from the drier and hotter thorn savanna of Alice (47.6% - A2 and B cytotypes), indicating that the presence of the compound chromosome might allow for better cold tolerance Brown *et al.* (1999). Although it is hard to establish whether cytotype is indeed responsible for climatic adaptation, there is some evidence for the correlation of the two (Taylor *et al.* 1994, Brown *et al.* 1999).

The parapatric distribution of these various cytotypes (Figure 1.1) raises the question as to whether they are reproductively isolated. Pillay *et al.* (1992, 1995) conducted laboratory-based breeding experiments where he mated individuals from Kamberg (cytotype A1) with individuals from Karkloof (cytotype A2) and found a significant reduction in successful matings of interpopulation crosses (47% success), compared to intrapopulation crosses (100% success). Also documented in these interpopulation crosses was a reduction in the number of offspring, smaller litter sizes as well as poor post-natal development (Pillay *et al.* 1995). The authors speculate that there is a breakdown of recognition cues (olfactory or auditory) that prevent successful mate recognition further contributing to the lowered reproductive success. There were higher levels of aggression in holding cages in which interpopulation crosses were performed than there were in the cages wherein intrapopulation crosses were performed (Pillay *et al.* 1995). Aggression might therefore also act as a premating isolation barrier. These findings strongly suggest the presence of both pre-mating isolation and, if successful mating occurs, lower fecundity following post-zygotic isolation ensured the populations remained divergent.

To find a genetic basis for distinction between the various cytotypes Taylor *et al.* (1992) analysed 24 protein coding loci of individuals from 12 different locations using allozyme

electrophoresis. The sampling range of this study encompassed all the described cytotypes. This revealed a global panmictic genetic structure for South African *O. irroratus* (F_{st} =0.37; D= 0.03), indicating high levels of gene flow between populations (Taylor *et al.* 1992). However, the low genetic distances and low levels of heterozygosity (H = 0.042 – 0.061), suggested that this marker does not evolve rapidly although a certain degree of genetic structuring among populations and cytotypes are evident.

Mitochondrial cyt *b* sequence data has proven more useful in detecting differences between the various cytotypes (Maree 2002, Taylor *et al*. 2009a, Engelbrecht *et al*. 2011). Overall, two monophyletic lineages, each containing two sub-clades within *O. irroratus* are evident. These subclades correspond to the described cytotypes of *O. irroratus*, with niche modelling revealing a correlation between these cytotypes and climate (Engelbrecht *et al.* 2011a). Studies based on cranial morphometrics did not show a clear distinction between the various cytotypic populations of *O. irroratus*, however it did find two differentiated groups consisting of samples from the Fynbos and the Thicket biomes and the other consisting of samples from the Grassland biome (Taylor *et al.* 2004, 2009a). The sequence divergence values, represented by uncorrected p-distances from the mtDNA cyt *b* gene, between these groups were sufficient to warrant the recognition of a new species, referred to as *O. auratus* (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a).

1.6 Chromosomal speciation theory

The biological species concept defines species as a group of interbreeding natural populations, reproductively isolated from other such groups (Mayr 1966). The role of chromosomal rearrangements in producing such reproductively isolated natural populations, has received much attention (White 1978a, b, King 1993, Rieseberg 2001, McAllister *et al.* 2008, Castiglia 2014). The foundation of this theory derives from the observation that species

often have different diploid numbers (King 1993), and the underlying assumption that this preceded speciation. However, this argument is counter balanced by evidence showing that some chromosomal rearrangements have little or no effect on fertility and consequently they are ineffective barriers to gene flow (Spirito 1998, Strasberg *et al.* 2009).

Two evolutionary scenarios have been proposed explaining how chromosomal rearrangements could lead to the formation of species (Figure 1.2). In the first instance, it has been widely observed that individuals heterozygous for chromosome rearrangements will be partially or totally infertile as result of segregation problems resulting in unbalanced gametes (Hybrid dysfunctional models, see Navarro *et al.* 1997, Rieseberg 2001). According to these models heterozygote carriers of chromosome rearrangements are underdominant (less fit than homozygotes carriers), therefore these chromosome rearrangements can act as genetic barriers to gene flow between populations with fixed chromosomal differences (Navarro and Ruiz 1997, Navarro *et al.* 1997, Rieseberg 2001). This model has however been criticised as evidence for strong underdominance was not common (Faria and Navarro 2010, Brown and O'Neill 2010). The mechanism as to how underdominant chromosome rearrangements can become fixed in a population is not clear, and when these underdominant chromosomal rearrangements are indeed weak enough to become fixed, they would not constitute strong enough barriers to gene flow to cause speciation (Spirito 1998, Rieseberg 2001, Strasberg *et al.* 2009). Nonetheless it is possible that such underdominant chromosomal rearrangements can become fixed in small interbreeding populations, through the effects of random genetic drift (Coluzzi 1982, Faria and Navarro 2010, Brown and O'Neill 2010) or meiotic drive (White 1978a, Rieseberg 2001). This would lead to the isolation and differentiation of various genetic subpopulations (Coluzzi 1982), enabling the persistence of floating chromosomal polymorphisms (Dobigny *et al.* 2017), as that documented in the vlei rat (Engelbrecht *et al.* 2011b).

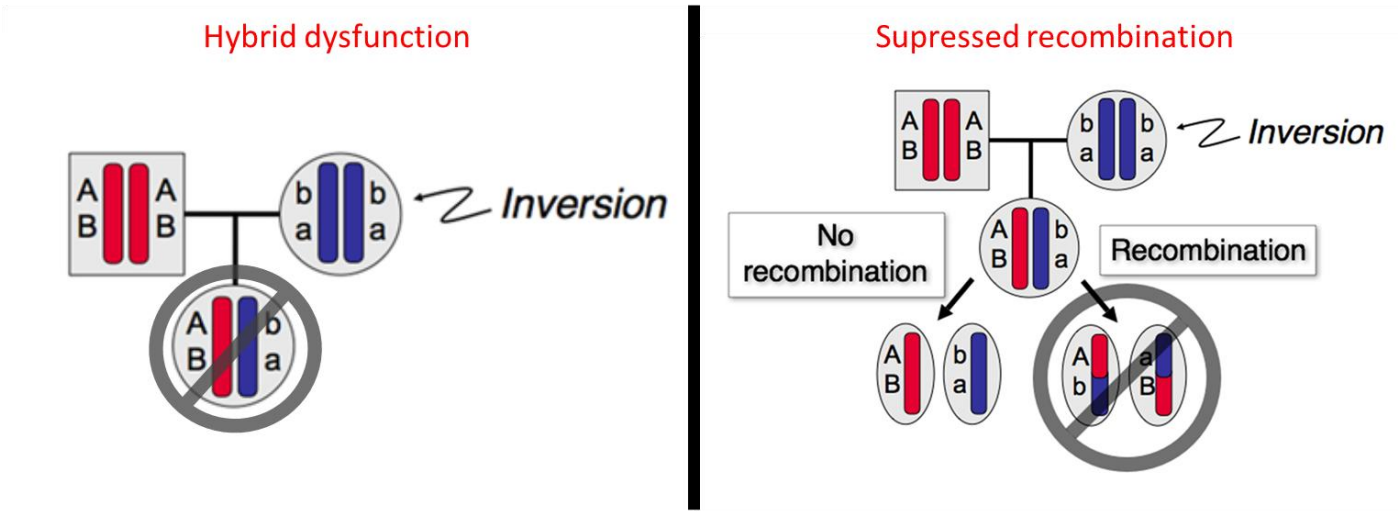


Figure 1.2. Diagram illustrating the two models of chromosomal speciation. In the hybrid dysfunction / sterility model heterozygote hybrids resulting from a cross between a normal and an inversion homozygote, would be underdominant and incapable of producing viable gametes (left). In the supressed recombination model hybrids resulting from a cross between a normal and an inversion homozygote, would only be able to produce viable gametes if recombination is supressed in the inverted regions. This would lead to the accumulation of incompatibilities and facilitate speciation (right). The diagram is from Kirkpatrick (2010).

The alternative model for chromosomal speciation proposes that gene flow may occur between species with different diploid numbers (supressed recombination model; Noor *et al.* 2001, Rieseberg 2001 – Figure 1.2). This model suggests that recombination occurs differently between rearranged chromosomal regions than it does between co-linear chromosomal regions. In effect, this model suggests suppression of recombination in rearranged regions and free wholesale recombination in collinear regions. A supressed recombination rate would cause a reduction of gene flow across rearranged genomic regions, as well as an accumulation of incompatibilities, ultimately leading to speciation (Farré *et al.* 2013). One of the first lines of evidence for this model of chromosomal speciation in mammals originated from studies on the Fat Sand Rat *Psammomus obesus* (Ashley *et al.* 1981), where they identified the presence of a pericentric inversion by calculating the relative position of the kinetochores (centromeric position of spindle fibre attachment) of two homologous sister chromatids. Here they noted

that crossing over does not occur in these inverted segments, clearly indicating the suppression of recombination. This implies the typical genetic consequences of a pericentric inversion, such as duplications and deletions will not occur (Colluzi 1982), and in this way meiotic irregularities will be avoided (Del Cerro *et al.* 1998, Ostberg *et al.* 2013, Ortíz- Barrientos *et al.* 2016). This was corroborated by data emanating from organisms such as *Anopheles* mosquitoes (Coluzzi 1982), Deer Mouse (*Peromyscus sitkensis*-Greenbaum and Reed 1984, Hale 1986), *Drosophila* (Navarro and Ruiz 1997, Navarro *et al.* 1997) and for primates (*Pan troglodytes* - Farré *et al* 2013 and *Macaca* – Ullastres *et al.* 2014). These studies essentially showed that pericentric inversions are indeed useful for understanding chromosome evolution and speciation. A reduction in gene flow and number of meiotic cross overs have been noted in the House Mouse *Mus musculus* (Nachman and Searle 1995, Piálek *et al.* 2005, Capilla *et al.* 2014) and the Common shrew *Sorex araneus* (Basset *et al.* 2006, Belonogova *et al.* 2018) as result of Robertsonian fusions and translocations, suggesting that inversions are not the only rearrangement that can lead to speciation. Against this background, I investigated how inversions and other rearrangements found in the vlei rat influenced chromosome recombination and pairing during meiosis.

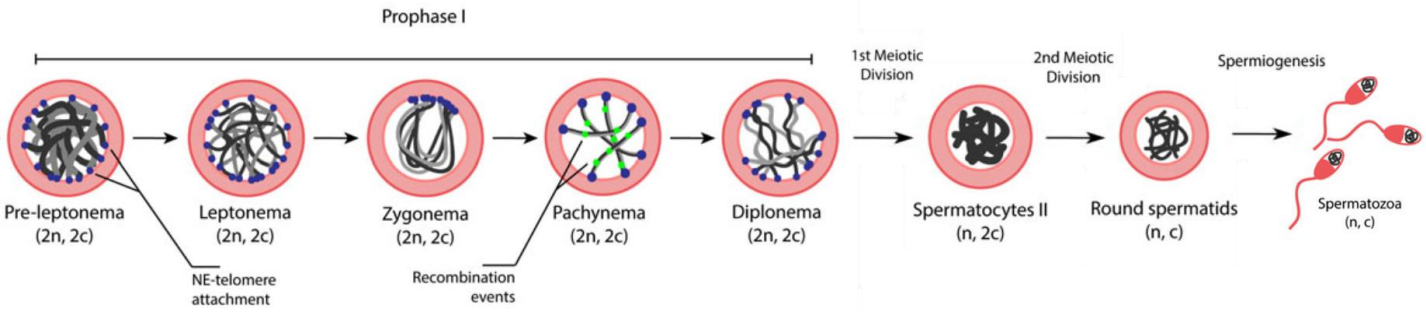


Figure 1.3. Schematic representation of male meiosis including the four differentiated stages of prophase I. The blue dots indicate attachment points of telomeres to the nuclear envelope and the green dots recombination events. The numbers in brackets indicate the

chromosome and chromatid count per cell type as either diploid 2n (2c) or haploid n (c). Adapted from Reig-Viader *et al.* (2016).

1.7 Chromosomal recombination

1.7.1 Gametogenesis and meiotic recombination

Meiotic cell divisions reduce the number of chromosomes from a diploid set (2n), to a haploid set (n) (Burgoyne *et al.* 2009). Two consecutive cell divisions (meiosis I and meiosis II) each comprising of four stages of a typical cell division: prophase, metaphase, anaphase and telophase (reviewed in Handel and Schimenti 2010, Capilla *et al.* 2016) will ultimately lead to the production of haploid (n) gametes.

Meiotic recombination occurs during prophase I, a process which is further subdivided into five differentiated stages (Figure 1.3): leptonema, zygonema, pachynema, diplonema and diakinesis (Handel and Schimenti 2010, Reig-Viader *et al.* 2016). During leptonema, homologous chromosomes will pair through the help of proteinaceous structures along chromosomes formed by cohesion subunits (REC8 and SMC1) as well as proteins of the Synaptonemal Complex (SC). The SC is a tripartite structure with axial elements, made up of Synaptonemal Complex Protein 2 and 3 (SYCP2 and SYCP3) connected by perpendicular filament proteins with the overlapping central element (Figure 2.2), Synaptonemal Complex Protein 1 (SYCP1) (Heyting, 1996; Page and Hawley 2004) which permits the alignment and pairing of homologous chromosomes. Meiotic recombination starts with the simultaneous formation of double strand breaks (DSBs), by the endonuclease protein SPO11 (Keeney *et al.* 1997; Romanienko and Camerini-Otero, 2000; Longhese *et al.* 2009). The double strand breaks are repaired at the zygonema stage of prophase I. During this stage, two parental homologs synapse, producing either crossovers (COs) or non-crossovers (NCOs) (Burgoyne *et al.* 2009). If COs form, a D-loop will be created that captures the second 3’ end of the

homologous chromosome, and after the DNA synthesis and ligation, a Double Holliday Junction (dHJ), with heteroduplex DNA flanking the DSBs site, is produced (Collins and Newlon 1994). Recombination is resolved at the pachynema stage. At this stage the SC is completely established and bivalent structures and COs are resolved by the repair pathway directed by proteins MutS Homologs 4 and 5 (MSH4 and MSH5- Figure 2.2) (that appear earlier at zygonema) and MutL Homologs 1 and 3 (MLH1 and MLH3) (Kneitz *et al.* 2000; Lipkin *et al.* 2002, Snowden *et al.* 2004). Homologous chromosomes start to segregate during diplonema, they continue to keep contact at CO sites (chiasmata structures) until anaphase is complete (Speed 1982). Prophase I is now complete and metaphase I will commence. During metaphase, the nuclear envelope disappears, chromosomes which are totally condensed, migrate to the equatorial plane of the cell. At anaphase I these chromosomes will separate and move from the equatorial plane to the poles. At telophase I, genetically different daughter cells, with haploid chromosome dotation (n) will be generated, as result of the genetic recombination process (Capilla *et al.* 2016)

The process of meiotic recombination (Figure 1.4) is important from an evolutionary perspective as it produces genetic variation (Kauppi *et al.* 2004), determines the effects of selection of polymorphisms (Smith and Haigh 1974, Dumont and Payseur 2011) and ensures proper alignment and disjunction of homologous chromosomes during meiosis (Petronczki *et al.* 2003, Borodin 2008, Wang *et al.* 2015).

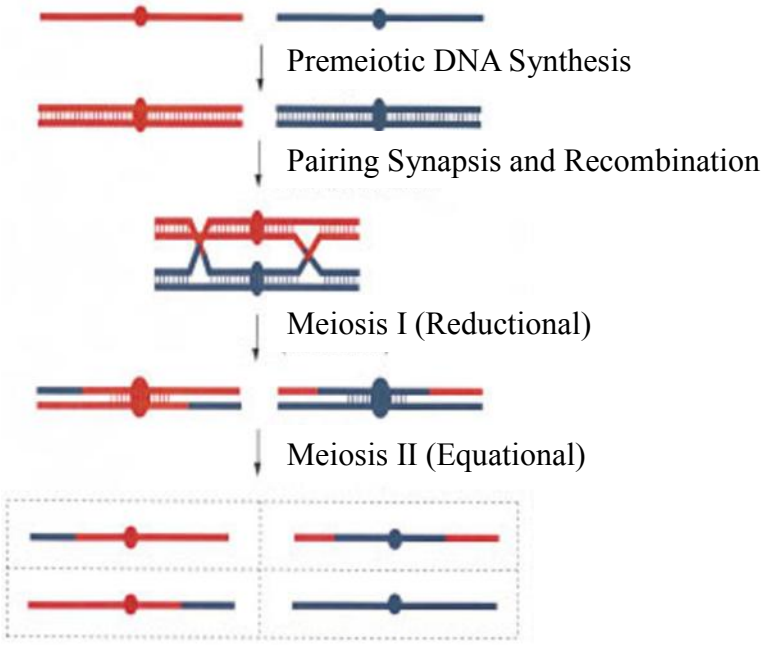


Figure 1.4. Simplified diagram illustrating how recombinant gametes are formed through pairing, synapsis and recombination of maternal and paternal lines throughout meiosis. Roeder (1997).

1.7.2 Crossovers and interference

Crossovers are non-randomly distributed among chromosomes (Carpenter 1988, Lynn *et al.* 2002, Fung *et al.* 2004, Kauppi *et al.* 2004) and appear to be tightly regulated (Börner *et al.* 2004). It is now known that these recombination events are highly localized, occurring in 'hot spots' whereas the bulk of the DNA is 'cold' (Paigen and Petkov 2010). The position of these 'hot spots' have also been found to affect chromatin structure in the modulation of DNA function, making it important in all mechanistic studies of population genetics and evolutionary processes of DNA function (Paigen and Petkov 2010). The distribution of crossovers is further characterised by two other features. Firstly, there is always at least one crossover or chiasma per pair of chromosomal homologs (Börner *et al.* 2004). This is referred to as the 'obligatory chiasma', which ensures the proper disjunction of homologous chromosomes during the first meiotic division (Bishop and Zickler 2004, Fung *et al.* 2004, Segura *et al.* 2013).

Secondly, if two or more crossovers occur along the same bivalent, they exhibit 'interference' which causes them to be widely spaced between each other (Roeder 1997). Interference prevents crossovers occurring close to each other. For one crossover to have such a repellent effect they must therefore generate some sort of crossover-discouraging signal that spreads for some variable distance, along the chromosome, on either side of the crossover (Hillers 2004). After detecting a higher frequency of crossover events in smaller chromosomes of *Saccharomyces cerevisiae*, than in larger ones (Kaback *et al.* 1992), it was noted that interference strength is inversely correlated with distance (Bishop and Zickler. 2004). Larger chromosomes therefore display more interference than small ones (Kaback *et al.* 1999).

1.7.3 Recombination and Speciation

The interaction between hybridization and recombination can determine the likelihood of speciation or persistence, of incompletely isolated species (Ortíz-Barrientos *et al.* 2002, 2016). Recombination can break apart genes and gene complexes through linkage disequilibrium (Servedio 2009), while at the same time allowing different genes or alleles to fix in the population (Ortíz-Barrientos *et al.* 2002). This implies recombination can split two adaptive genes, which will facilitate the persistence of species, or alternatively, it could split two genes allowing the persistence of species, then again favouring the divergence of species (see Mayr 1966, Felsenstein 1981). These studies generally focus on regions of chromosomes that have been rearranged throughout the evolutionary history of the species under study, as chromosome rearrangements are known to compromise recombination rates (Rieseberg 2001, Dumont *et al.* 2009). Therefore, recombination rates may provide valuable insight into how species and general biodiversity is generated (Butlin 2005, Capilla *et al.* 2016).

Examination of recombination rates has also provided some valuable insights into sex chromosome evolution and has been implemented in the description of its origin. Charlesworth

and Charlesworth (1978) states that sex chromosomes evolved from a pair of autosomes by a selective pressure to link two sex determination loci, the one promoting maleness and the other to supress femaleness. These two sex determination loci eventually became permanently linked when recombination became supressed in this region. The suppression of recombination allowed for the accumulation of deleterious mutations, caused the Y-chromosome to degenerate then ultimately give rise to the heteromorphic sex chromosome pairs. The suppression of recombination between sex determining genes was therefore the most crucial event in the evolution of sex chromosomes (Ming *et al.* 2008).

1.7.4 Recombination rates in mammals

Recombination rates of several mammals such as humans (Hellman *et al.* 2003), rodents (Jensen-Seaman *et al.* 2004, Dumont and Payseur 2008, 2011), monkeys (Garcia-Cruz *et al.* 2011), chimps (Farré *et al.* 2013), dogs (Basheva *et al.* 2008) and bovids (Ruiz-Herrera *et al.* 2017) has been assessed. In these studies, recombination rate was normally quantified by the number of MLH1 foci, which directly reflects the number of crossovers (Anderson *et al.* 1999). From these, studies focusing on rodents were the most abundant. They were found to have a remarkably low rate of recombination, in comparison to other mammalian taxa (Dumont and Payseur 2008, 2011, Segura *et al.* 2013). The low rate of recombination of rodents may to an extent be a consequence of their unique karyotypes, which are generally comprised of acrocentrics. Smaller chromosomes have limited space for crossovers and hence display lower recombination rates than the chromosomes of other mammals which have larger proportion of bi-armed chromosomes (Dumont and Payseur 2008, Segura *et al.* 2013). Dumont and Payseur (2011) speculates that a low rate of recombination may be selectively favoured by natural selection. A low rate of recombination may in this instance prevent the decoupling of linked high fitness alleles. Alternatively, a low rate of recombination could prevent the establishment of higher fitness allelic combinations due to linkage effects, slowing evolution

by rendering it dependent on smaller scale change i.e. point mutations. Similarly, selection could favour an increase in recombination rate, to facilitate the removal of deleterious alleles, especially in the instance of laboratory bred plants and animals, which may suffer under inbreeding depression.

Recombination rates can to a great extent be influenced by chromosomal rearrangements such as Robertsonian fusions and inversions. These types of rearrangements could significantly alter the recombination landscapes causing changes in genetic populations, even in the face of gene flow (Dumas and Britton-Davidian 2002, Capilla *et al.* 2014, Belonogova *et al.* 2018). These rearrangements are both present in vlei rat, as floating polymorphisms.

1.8 Rationale

The karyotypic variation documented for this species has raised the question as to whether *O. irroratus* is one species. Recent studies have found that there is cryptic speciation occurring within *O. irroratus* s.l. (Engelbrecht *et al.* 2011a). Here I explore whether chromosomal rearrangements facilitated divergence within this species complex. Chromosome rearrangements can either lead to speciation through hybrid dysfunction, supressed recombination or an interplay of the two (Brown and O'Neill 2010, Faria and Navarro 2010). The study of chromosomal behaviour during gamete formation will be very informative in assessing the effects of chromosome rearrangements on fertility and structuring reproductively isolated populations. Vlei rats serve as an ideal model to investigate how segregation of gametes occur in heterozygous individuals, due to their high levels of intra-chromosomal variation (Contrafatto *et al.* 1992a, b) and the fact that the geographic distribution of these cytotypes has been well documented (Taylor *et al.* 2000, Rambau *et al.* 2001, Engelbrecht *et al.* 2011a).

1.9 Research aims and Objectives

The primary objective of this study was to investigate recombination rates and chromosome rearrangements in previously documented populations and new populations of *O. irroratus* s.l. This was achieved through karyotyping and molecular cytogenetics, examination of chromosome pairing and recombination during pachytene of meiotic prophase I, in both carriers of rearrangements (pericentric inversions, Robertsonian fusions and tandem fusions), and non-carriers of chromosome rearrangements (standard populations). Additionally, specimens were sequenced to establish and compare genetic divergence (uncorrected p-distances) between populations and individuals that carry chromosome rearrangements in their karyotypes and those that do not. Thus, the aims of the study were twofold:

(1) To establish whether there is a significant difference in pairing and recombination between the two different cytotypes as well as carriers and non-carriers of inversions, Robertsonian fusions, and tandem fusions in both respective cytotypes, as identified by G-banding.

Null Hypothesis: There is no difference in recombination rates between carriers and standard populations of *O. irroratus* s.l.

Expected outcome: To elucidate how meiotic recombination enables the persistence of deleterious chromosomal rearrangements (such as pericentric inversions) and polymorphic Robertsonian rearrangements, in different vlei rat populations, as well as how it differs between populations of chromosomally variable vlei rats.

(2) To determine whether chromosomal reorganizations between lineages of *O. irroratus* s.l. (2n= 24 vs 2n= 28-31), is linked to the divergence between these respective lineages?

Null hypothesis: There is no significant difference among the mtDNA lineages of *O. irroratus* s.l.

Expected outcome: To shed light on whether DNA sequence divergence is similarly reflected amongst the lineages of *O. irroratus* s.l.

Chapter 2: Revisiting cytogenetic variation of *Otomys irroratus*, with insights from synaptonemal complex data

2.1 Introduction

Two main chromosomal blueprints are described for *Otomys irroratus* s.l. (OIR). They are separated by the presence of a tandem fusion chromosome consisting of OIR chromosomes 7, 8 and 12, producing two cytotypic lineages with a 2n= 24 (with tandem chromosome) and 2n= 28 (without tandem chromosome; Rambau *et al.* 2001). Furthermore, pericentric inversions (Robinson and Elder 1987; Taylor *et al.* 2009a, Engelbrecht *et al.* 2011b), Rb fusions (Contrafatto *et al.* 1992b; Rambau *et al.* 2001) and heterochromatic additions and deletions have been described in *O. irroratus*. The pericentric inversions in *O. irroratus* karyotypes occur on chromosomal pairs 1, 2, 4, 6 and 10 (Figure 2.1). The inversion on OIR1 has only been detected in heterozygous state in three specimens of a Kroomie population (Eastern Cape) and OIR2 has been detected in Grahamstown in the Eastern Cape (Taylor *et al.* 2009a). The inversion on OIR4 has been found in the homozygous and heterozygous state in four Western Cape populations (Porterville, Oudtshoorn, Stellenbosch and Beaufort West) and in Grahamstown. The inversion on OIR6 has been found in Constantia, Porterville, Stellenbosch, Beaufort West, Van Rhynsdorp (Western Cape) as well as in the Eastern Cape – Tsitsikamma, Somerset East, Kroomie, Alice and Grahamstown, also in homozygous and heterozygous states. The inversions on OIR10 have only been found in heterozygous state, in Oudsthoorn, Stellenbosch and Beaufort-West (Figure 2.1). These inversions were detected by G-banding, C-banding (Robinson and Elder 1987, Contrafatto *et al.* 1992 a, b) and FISH, using selected whole chromosome paints developed for the bush karoo rat, *Myotomys unisulcatus* (Engelbrecht *et al.* 2011b) and appears to have the highest prevalence and diversity in the Eastern Cape roughly in the centre of its continuous distribution along the south and east coast

of South Africa. This is consistent with the findings of early studies by da Cunha and Dobzhansky (1954) which found that the central parts of a species distribution are the most differentiated with the highest heterozygosity, compared to the more marginal or peripheral populations (cited in King 1993).

Figure 2.1. Distribution of rearrangements documented in *O. irroratus* s.l. Superscript number represents inversion on respective OIR chromosome indicated by letter i. Localities investigated in this study, as well as instances where rearrangements were documented in a new locality are listed in bold, the remainder indicates where rearrangements were documented in previous studies (Robinson and Elder 1987, Rambau *et al.* 2001, Engelbrecht *et al.* 2011b). The blue circle marks Sandile's Rest where no rearrangements were found and Rb1/6 indicates Robertsonian rearrangement found at Mbotyi.

Inversions in rodents (Volobouev *et al.* 2001, Dobigny *et al.* 2005), mosquitoes (White *et al.* 2007a, b) and even yeast (Leducq *et al.* 2016) are generally known to have detrimental fitness consequences to the carrier as it leads to the production of unbalanced gametes (Kirkpatrick 2010). Similarly, Rb fusions in mice (Castiglia and Capanna 2000, Castiglia *et*

al. 2015) and other rodents (Basheva *et al.* 2014), have been shown to cause meiotic breakdown due to monobrachial homology (Baker and Bickham 1986). The intraspecific variation resulting from rearrangements such as inversions and Rb fusions could lead to incipient speciation. Both these classes of rearrangements are present in *O. irroratus* karyotypes, but the mechanism/s which allows these seemingly deleterious rearrangements to persist is unknown. It is thought that these rearrangements exist as floating polymorphisms in *O. irroratus* populations, meaning they probably have a minor effect on fertility (Engelbrecht *et al.* 2011b). If this was not the case and these rearrangements conferred a degree of underdominance, their abundance could be explained by population demographics, drift, meiotic drive or selective advantage effects. Alternatively, the high prevalence and persistence of inversions in polymorphic state could suggest that overriding mechanisms may be at play. As recombination occurring between non-homologous (standard and arranged chromosomal homologue) could lead to duplications, deletions and hence meiotic irregularities, (Colluzi 1982, King 1993, Ostberg *et al.* 2013), it has been proposed that the suppression of recombination in these rearranged chromosomal regions would prevent the adverse genetic consequences caused by of these rearrangements (Del Cerro *et al.* 1998, Ortíz- Barrientos *et al.* 2016). The analysis of chromosome behaviour during the prophase I stage of meiosis could shed light on this matter.

During prophase I chromosomes are arranged into DNA loops (Ruiz-Herrera *et al.* 2017) which are attached to the synaptonemal complex, a tripartite structure with axial elements (Figure 2.2), made up of Synaptonemal Complex Protein 2 and 3 (SYCP2 and SYCP3) connected by perpendicular filament proteins with the overlapping central element, Synaptonemal Complex Protein 1 (SYCP1) (Heyting 1996; Page and Hawley 2004). This structure permits the alignment and pairing of homologous chromosomes during leptonema, the first sub stage of Prophase I. Meiotic recombination starts with the simultaneous formation

of double strand breaks (DSBs), by the endonuclease protein SPO11 (Keeney *et al.* 1997; Romanienko and Camerini-Otero, 2000; Longhese *et al.* 2009). The DSBs are repaired at the zygonema stage of prophase I. During these stage two parental homologs synapse, producing either crossovers (COs) or non-crossovers (NCOs) (Burgoyne *et al.* 2009). Recombination is resolved at the pachynema stage, then the SC is completely established, and bivalent structures and COs are resolved by the repair pathway directed by proteins MutS Homologs 4 and 5 (MSH4 and MSH5) (that appear earlier at zygonema) and MutL Homologs 1 and 3 (MLH1 and MLH3) (Kneitz *et al.* 2000; Lipkin *et al.* 2002, Snowden *et al.* 2004). The recombination process is concluded during diplonema, when homologous chromosomes segregate while maintaining contact at CO sites (chiasmata structures) until anaphase is complete (Speed 1982).

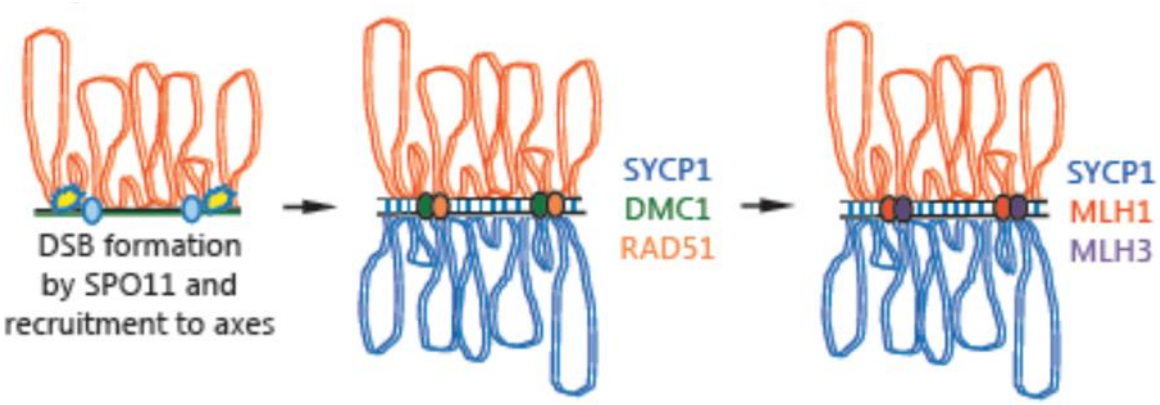


Figure 2.2. Diagram illustrating the proceedings of crossing over and recombination during meiotic prophase 1, with some key proteins. Double strand breaks (DSBs) are initiated by the endonuclease SPO11 during leptonema (left) and pairing of homologous chromosomes start. The synaptonemal complex is formed at this stage through SYCP2 and SYCP3 (green and black horizontal lines representing the chromosomal axes where DNA loops are anchored). The DSBs are recognized and the binding of repair proteins DMC1 and RAD51 takes place (middle). At zygonema the homologous chromosomes are completely paired (represented by orange and blue loops attached to synaptonemal complex) by the central elements SYCP1 (ladder like structure between synaptonemal complexes) and synapsis commence. During pachynema (right) synapsis is completed and resolved into crossovers marked by mismatch repair proteins MLH1 and MLH3. Adapted from Capilla *et al.* (2016).

Meiotic recombination produces new genetic variants that can significantly alter the genomic landscape of animals, hence it has been regarded as key factor for consideration in evolutionary studies (Capilla *et al.* 2016). By using antibodies specific for recombination proteins such as MLH1, SYCP3, CENPC (centromeric protein), H3K9me3 (histone 3 trimethylated at lysine 9- showing heterochromatin, Hublitz *et al.* 2009) amongst others, immunostaining allows for the visualization of the proceedings of recombination in germ cells (Anderson *et al.* 1999, Manterola *et al.* 2009, Ullastres *et al.* 2014). The proteins targeted by these antibodies have remained largely conserved which makes comparisons amongst taxa possible (Segura *et al.* 2013). This has led to an increase in attention given to these antibodies as its value in the direct observations of chromosome rearrangements and meiotic consequence became recognized (see review Capilla *et al.* 2016).

Thus far studies have revealed that chromosome rearrangements such as Rb fusions (Bidau *et al.* 2001, Merico *et al.* 2003; Capilla *et al.* 2014, Belonogova *et al.* 2018) and inversions (Navarro and Ruiz 1997, Farré *et al.* 2013, Wadsworth *et al.* 2015) can change the distribution and frequency of crossovers and recombination events. In the House Mouse, chiasma repatterning and suppression has been documented in karyotypes with higher degrees Rb fusion/fission restructuring (Bidau *et al.* 2001, Dumas *et al.* 2015). The change in recombination patterns could in such a case disrupt supergenes and influence the symmetry of heterozygous meiotic configurations, leading to gametic imbalances and inviability. It is speculated that an inversion has driven the speciation of corn borer moths, as it supressed recombination in hybrids, housed adaptive genes within the rearranged segments and then facilitated sequence divergence (Wadsworth *et al.* 2015). In a similar chromosome rearrangements, could act as recombination suppressors and drive the chromosomal speciation of non-allopatric species such as the *O.irroratus* s.l. (Faria and Navarro 2010, Brown and O'Neill 2010).

Advances in molecular cytogenetic techniques such as immunostaining, has allowed for the first-hand visualization of meiotic chromosomes of different evolution taxa. This largely includes the use of antibodies, which targets certain key proteins involved in the meiotic process. The vlei rat, *O.irroratus* is the ideal model candidate for immunostaining as the meiotic consequences of the vast variety of chromosome rearrangements present in its karyotypes could then be directly assessed. This would provide invaluable insights into the persistence of underdominant chromosomal rearrangements in *O. irroratus* populations, and how it influences evolution in the species. The aims of this study are:

1- To investigate and compare chromosomal pairing and recombination in *O.irroratus* s.l. specimens with and without chromosome rearrangements through the use of immunofluorescence of meiotic proteins. The reference population was the standard karyotype with 2n= 28 (without B chromosomes and without chromosomal rearrangements).

2- To revisit and discuss the chromosomal variation documented in *O. irroratus* s.l. and shed light on the status and persistence of underdominant chromosomal rearrangements found in the species complex.

2.2 Materials and Methods

2.2.1 Sampling and tissue culture

A total of 37 samples of *O. irroratus* were collected from the Eastern- and Western-Cape provinces of South Africa (Figure 2.1, Table 2.2). Collection and handling of sample material took place according to acceptable international guidelines as described in Sikes and Gannon (2011). Tail and ear biopsies were taken and fibroblast cultures were established following standard cytogenetic protocol. Cultures were grown in media consisting of Dulbecco's Modified Eagle Medium (DMEM, GIBCO) enriched with 15% foetal calf serum buffer (FCS; GIBCO) and AmnioMAX (GIBCO), in incubation chambers maintained at 37°C and 5 % CO_2 (Schwarzacher and Wolf, 1974). Fibroblast cells were harvested following standard cytogenetic protocol (Schwarzacher and Wolf, 1974). This involved treatment in hypotonic solution (0.75 M KCl) after which cells were fixed in a 1:3 acetic acid: methanol solution. Chromosome banding techniques followed procedure as set out in Seabright (1971) and Sumner (1972; 1990). For G-banding, metaphase chromosomes were digested in trypsin (2.5 % in 1 X Phosphate buffered saline-PBS) for ~20 seconds, before staining with 5% Giemsa in 0.025 M KH_2PO_4 for 8 minutes (Seabright 1971). C-banding was also done to identify the position of constitutive heterochromatin within chromosomes (Sumner 1990). This involved treatment in saturated barium hydroxide $Ba(OH)_2$ solution for 1-3 minutes and washes in 2XSSC at 55 °C, followed by staining with 2% Giemsa. Images were captured in the Genus system version 3.7 (Applied Imaging Corp., Newcastle, UK) with a CCD camera mounted on an Olympus BX 60 epiflourescence microscope. Chromosomes were then arranged into karyotypes based on size and morphology, following the convention used by Robinson and Elder (1987).

2.2.2 Testes preparation and immunofluorescence

The testes of male samples were excised and stored in liquid nitrogen immediately after animals were sacrificed (SU-ACUD15-00076). The protocol for chromosomal spreading roughly followed that set out by Segura *et al.* (2013). Testes material was minced thoroughly in PBS before treatment with 1% Lipsol in a humid chamber at room temperature for 14 minutes. Thereafter 4% paraformaldehyde solution was added to the outer border of the liquid suspension and this was again incubated for 30 minutes. After incubation the solution was left to dry for ~2 hours at room temperature followed by three washes in 1% Agepon for 1 minute each. Slides were DAPI (4',6 diamidino-2-phenylindole) stained and a coverslip was mounted with antifade (Vectashield). The quality of the slides (density and spreading of cells) was assessed under fluorescent microscopy, and then either used immediately for immunofluorescence, or frozen at -20°C.

For immunostaining, slides were submerged in distilled water for 10 minutes to remove the coverslip, followed by a wash in a blocking solution (PBS/Tween 0.05%). A volume of 100 µl of the primary antibodies -prepared in dilution 1:100 with the blocking solution was then added to the slide and incubated in a humid chamber overnight at 4°C. After incubation slides were washed twice in blocking solution for 5 minutes each at 37°C before 200 µl of the appropriate secondary (prepared in dilution 1:200), was added onto the slide and incubated at 37°C for 1 hour. Thereafter slides were washed twice in blocking solution and twice in PBSX1 for 5 minutes each at room temperature, before being DAPI stained and mounted in antifade with a coverslip.

These secondary antibodies were labelled with fluorescent dyes (e.g. Cy3 and FITC) which makes the localization of the primary antibodies possible through epifluorescent microscopy. The primary antibodies are normally manufactured in laboratory animals such as

mice and rabbits, which can in turn only be bound by a secondary antibody which are made in the same animal class e.g. αRabbit SYCP3 detected with Goat αRabbit Cy3.

2.2.3 Optimization of immunofluorescence protocol

Besides the various antibody combinations tried (Table 2.1), several other aspects of the original protocol by Segura *et al.* (2013) were modified, in attempt to make the hybridization of the recombination mismatch repair antibody (MLH1) and centromeric antibody CENPC possible. Various concentrations of both primary and secondary antibodies were explored (see Table 1 in appendix). Where the original protocol stated that the testes should be minced in ~20 µl of PBS, I also experimented with 40, 60, 100, 150 and 200 µl of PBS as I suspected that the spermatocyte concentration and spreading could inhibited hybridization of MLH1. Thereafter, changes to the original 8-minute Lipsol treatment time were introduced, modifying incubation times ranging from 1 minute to 16 minutes. Following the Lipsol treatment a diluted 1% paraformaldehyde fixative rather than a 4% paraformaldehyde was tried in order to assess whether different concentrations would have any significant impact on the fixation of the testes material (i.e. concentration affected fixation of material). The testes material would be firmly fixed when using paraformaldehyde at a high concentration. The antibodies were prepared with either 4XSSC or PBS/Tween. The manipulations listed here were done for each of the various primary antibodies which were paired with the all the available secondary antibodies for detection (Table 1 in appendix, Table 2.1).

Table 2.1: List of primary and secondary antibodies combinations used in this investigation, with its given suppliers. The primary antibodies bind directly to the proteins and antigens involved in meiosis, as explained above, whereas the secondary antibodies binds to these respective primary antibodies.

Primary	Secondary	Success
αRabbit SYCP3- Abcam	αRabbit Cy3- Abcam	Worked
	αRabbit FITC-Calbiochem	Worked
αRabbit SYCP3-Calbiochem	αRabbit Cy3- Abcam	Worked
	αRabbit FITC-Calbiochem	Worked
αMouse CENPC - Abcam	Donkey αMouse FITC- Abcam	Did not work
	Goat anti Mouse FITC- Abcam	Did not work
	αMouse FITC – Jacksons lab	Worked on 1 specimen
	αMouse Texred- Calbiochem	Did not work
αRabbit H3k9- Abcam	αRabbit Cy3- Abcam	Worked
	αRabbit FITC- Calbiochem	Worked

2.3 Results

2.3.1 Karyotypic descriptions per locality

A total of 30 karyotypes (2n= 24 – 30) was examined from 8 different localities (Table 2.2, see appendix). These included two different cytotypes, one comprised of 6 – 9 bi-armed chromosomal pairs with heterochromatic short arms, and the other of acrocentric chromosomes. Of these karyotypes, 17 specimens had rearrangements (excluding heterochromatic loss and B chromosome presence). Inversions were detected on OIR1, 4, 6, 7 and 10. A Rb fusion (OIR1/6) was found in one specimen from Mbotyi (Figure 2.3).

(a) Middelvlei Stellenbosch

The karyotypes of three specimens from this locality displayed a 2n= 28. These karyotypes consisted of nine pairs of submetacentric chromosomes (OIR1 – 4 and OIR6 – 10), four acrocentric chromosomal pairs (OIR5, 11 – 13), an acrocentric Y- chromosome and a submetacentric X chromosome (see Appendix). Inversions on OIR4, 6, 10 as well as a heterozygous polymorph OIR7 were also detected in one specimen (Figure 2.3). This heterozygous polymorphism is the result of a pericentric inversion.

(b) Somerset-East

The karyotypes of six specimens were examined and 2n was retrieved as 2n= 28 for all but one specimen, which had 2 B chromosomes resulting in a 2n= 30 (Figure 2.3). The karyotypes consisted of bi-armed chromosome pairs OIR1 – 7 and OIR9 – 10, acrocentric pairs in OIR8 and 11 – 13, a small acrocentric Y and a submetacentric X- chromosome (see Appendix). Inversions on OIR1, 6 and 10 were detected in this locality.

Table 2.2: *Otomys irroratus* specimens analysed in this study with the rearrangements documented per locality: N indicates the number of individuals gathered per sex indicated by ♂ and ♀, 2n (FN) shows the diploid chromosome numbers and number of fundamental chromosome arms of the specimens from a given locality, Inv 1,4,6,7 and 10 denotes inversions on respective chromosomes Rb 1/6 indicates where the Robertsonian fusion between OIR 1 and 6 has been documented and B chromosome indicates the amount of b chromosomes found. Presence indicated by (+) and absence (-). Chromosome rearrangement documented in a locality indicated by *.

Locality/ Coordinates	N	2n (FA)	Inv 1	Inv 4	Inv 6	Inv 7	Inv 10	Rb fusion	B chromosome
Stellenbosch, Western Cape 33°55’49’’S 18°49’44’’ E	3♂, 5♀	28 (42,46)	-	+	+	+*	+	-	-
Somerset-East, Eastern Cape 32°42’75’’S 25°38’55’’ E	5♂, 2♀	28-30 (44 – 49)	+*	-	+	-	+*	-	1-2
Kroomie, Eastern Cape 32°45’76’’S 26°23’63’’ E 32°47’19’’S 26°28’21’’ E	2♂, 7♀	28,29 (44 – 50)	+	-	+	+*	-	-	1
Kenton on Sea, Eastern Cape 33°34’05’’ S 26°34’25’’ E	1♂, 3♀	28 (46,47)	-	+*	+*	-	-	-	-
Fort Fordyce, Eastern Cape 32°40’86’’S 26°28’94’’ E 32°39’96’’S 26°29’37’’ E	1♂, 2♀	28 (45,48)	+*	-	+*	+	-	-	-
Grahamstown, Eastern Cape 33°19’19’’ S 26°31’04’’ E	1♀	28 (47)	+*	+*	+	-	+	-	-
Sandile’s Rest, Eastern Cape 32°40’06’’S 27°17’27’’ E	3♂	30 (33 – 37)	-	-	-	-	-	-	-
Mbotyi, Eastern Cape 31°25’22’’S 29°43’47’’ E	1♂, 1♀	24,30 (32,37)	-	-	-	-	-	1/6*	-

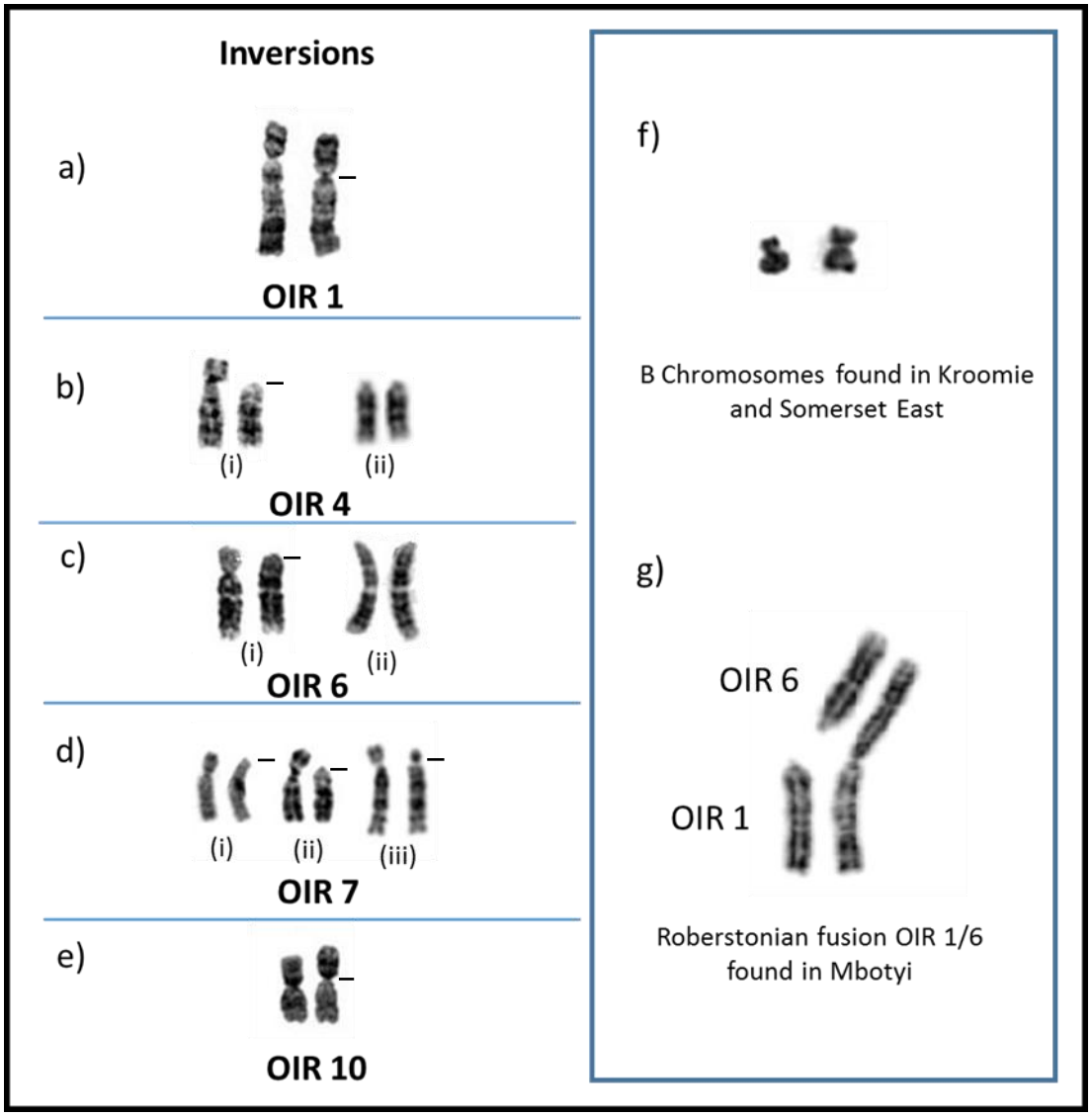


Figure 2.3. Structural chromosomal rearrangements found in specimens analysed in this study:(a) Heterozygous inversion OIR1 (N= 6) found in Somerset-East, Kroomie, Fort Fordyce and Grahamstown. (b) Inversion OIR4 (N= 5) found in Stellenbosch, Kenton on Sea and Grahamstown in heterozygous (i) and homozygous (ii) states. (c) Inversion OIR 6 (N= 11) found in Stellenbosch, Somerset-East, Kroomie, Kenton on Sea, Fort Fordyce and Grahamstown in heterozygous (i) and homozygous state (ii). (d) Heterozygous inversion on OIR7 (N= 3), from Fort Fordyce (i), Kroomie (ii) and Stellenbosch (iii). (e) Heterozygous inversion OIR10 (N= 4) found in Stellenbosch, Somerset-East and Grahamstown. The standard chromosome (without rearrangements) states are shown on the left with its inverted homologue on the right, with the altered centromeric position indicated by the dash. (f) B chromosomes found in Kroomie and Somerset-East. (g) Robertsonian fusion chromosome found in Mbotyi specimen, with its acrocentric homologues.

(c) Kroomie

All individuals had a 2n= 28 except for one individual which had one B chromosome, 2n= 29. The karyotypes also consisted of bi-armed chromosome pairs OIR1 – 7 and OIR9 – 10, acrocentric pairs in OIR8 and 11 – 13, a small acrocentric Y and a submetacentric X-chromosome. In this locality inversions were found on OIR1, 6 and 7. In one specimen, the inversion on OIR6 was in the homozygous state (Figure 2.3).

(d) Grahamstown

The karyotype of one female individual had a 2n= 28 and consisted of bi-armed chromosome pairs OIR1 – 7 and OIR9 – 10, acrocentric pairs in OIR8 and 11 – 13, and submetacentric X-chromosomes. This is the only individual out of all the other samples, which carried more than 3 rearrangements, those being on OIR1, 4, 6 and 10 (Figure 2.4a and b).

(e) Kenton on Sea

The karyotypes of four individuals all had 2n= 28 and consisted of bi-armed chromosome pairs OIR1 – 7 and OIR9 – 10, acrocentric pairs in OIR 8 and 11 – 13, submetacentric X chromosomes and small acrocentric Y- chromosomes. Inversion rearrangements have been found on OIR 4 and 6. One individual carried the inversion on OIR 4 in the homozygous state.

(f) Fort Fordyce

Two karyotypes both had 2n= 28 and consisted of bi-armed chromosome pairs OIR1 – 7 and OIR9 – 10, acrocentric pairs in OIR8 and 11 – 13, submetacentric X chromosomes and acrocentric Y chromosomes. The newly identified inversion rearrangement on OIR7 was detected along with those on OIR1 and 6.

(g) Sandile's Rest

The three individuals studied had the acrocentric karyotypes characterized by the A2 cytotype. A diploid number of 2n= 30 was retrieved for all specimens. The chromosomal pairs OIR9 and 10, as well as the X chromosome were bi-armed, whereas the remainder of the karyotypes all consisted of acrocentrics (see Appendix).

(h) Mbotyi

Two specimens had acrocentric karyotypes, one with a 2n= 24 and the other with a 2n= 30. The typical metacentric morphology of OIR9 was not evident for these specimens. The karyotype of the specimen with 2n= 30 were identical to that described for Sandile's Rest (see Appendix), while the individual with the 2n= 24 on the other hand, carried a Robertsonian fusion product between OIR1 and 6, in heterozygous state (Figure 2.3).

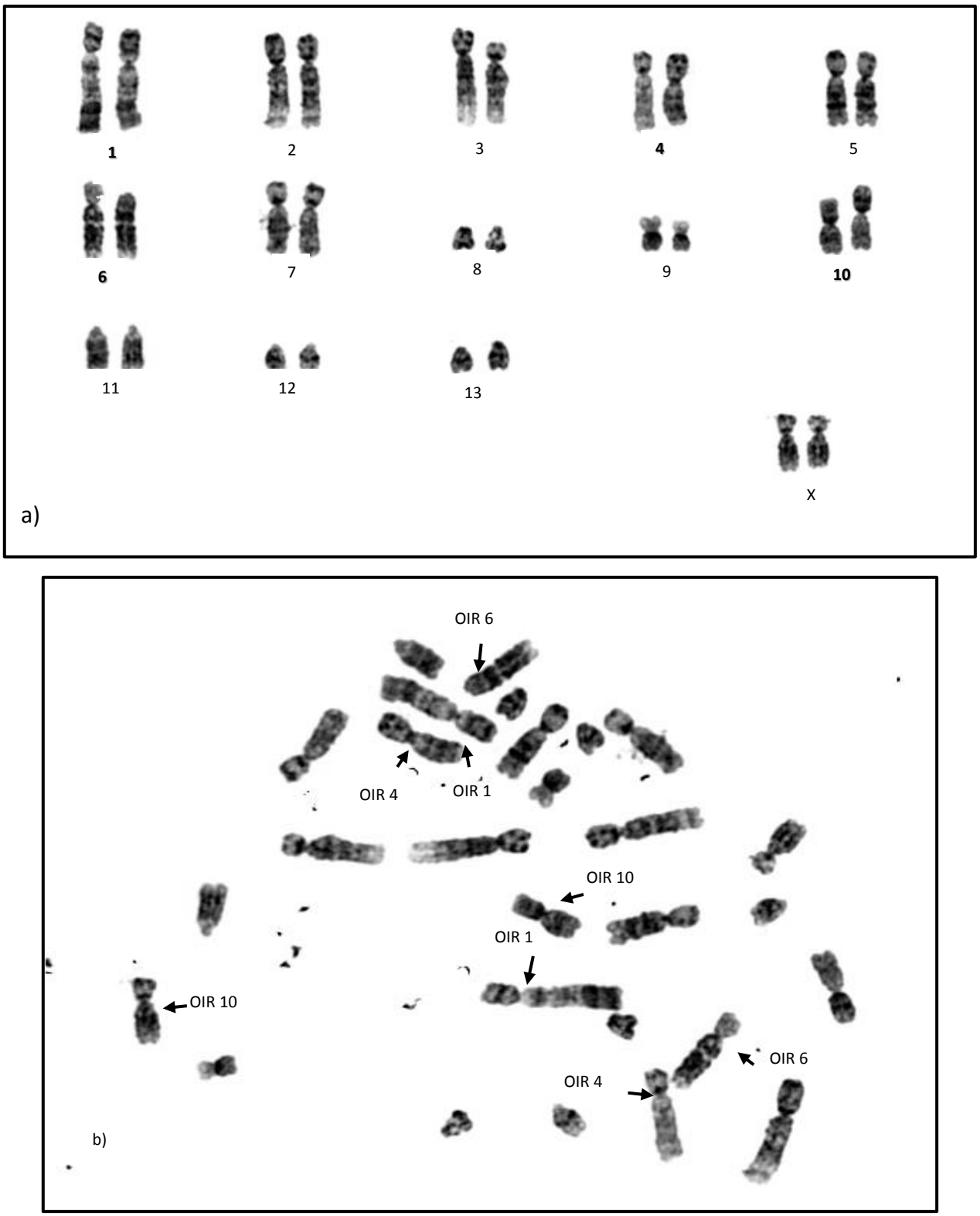


Figure 2.4. (a) Karyotype of individual from Grahamstown 2n= 28, which has inversions on OIR1, 4, 6 and 10 in heterozygous condition. Chromosomal pairs numbered in bold are heterozygous for a rearrangement with inversion breakpoints indicated by the dash. (b) The chromosome spread of individual a). Arrows point to position of centromeres (where inversion took place).

2.3.2 Meiotic data

The SYCP3 antibody was successfully hybridized to 11 male specimens from Stellenbosch (N= 1), Somerset-East (N= 4), Kroomie (N= 2), Kenton on Sea (N= 1), Fort Fordyce (N= 1), Sandile's Rest (N= 1) and Mbotyi (N= 1). A minimum of 60 cells were examined across three slides, for each specimen. A strong hybridization signal was clear throughout analyses of all specimens (Figure 2.5), which indicates that the axial elements of the synaptonemal complex remained intact, despite chromosomal rearrangements. Furthermore, no pairing anomalies were visible (even in chromosomes with putative rearrangements) as the lateral elements of the synaptonemal complexes were properly synapsed and presented as bivalents. The number of bivalents were consistent with the 2n (see Table 2.3). From the data gathered no points of crossovers could be identified between homologous chromosomes, and thus no conclusions could be drawn on the rates of recombination in *O. irroratus* karyotypes. Interestingly, no inversion loops were detected, even in specimens that appeared to have inversions. The CENPC antibody, which only hybridized successfully in one specimen, revealed a double signal in two bivalents of a Somerset- East specimen, giving the appearance of 2 centromeres per chromosome (Figure 2.5e, f) although this was not evident from the G-banding data. All other attempts to hybridize and visualize the MLH1 and CENPC antibodies in the rest of specimens were unsuccessful, despite countless efforts (see Table 1 of the Appendix).

Table 2.3: Summary of SYCP3 results from N= 11, representing seven localities. The locality and 2n is given for each specimen along with the number of paired chromosomes (bivalents) during meiotic prophase. The rearrangements represented by these karyotypes included inversions on OIR1, 4, 6 and 7, and the presence of B-chromosomes.

Locality,2n	**# Bivalents**	**Rearrangements**
Stellenbosch, 28	14	Heterozygous Inv 6
Somerset East, 28	14	Homozygous Inv 6 / Standard
Somerset East, 28	14	Heterozygous Inv 4 and 6
Somerset East, 28	14	Heterozygous Inv 1 and 6
Somerset East, 30	15	Heterozygous Inv 4 and 6 + 2 B chromosomes
Kroomie, 28	14	No rearrangements
Kroomie, 29	14 + 1 univalent	Heterozygous inv1 + 1 B chromosome
Kenton on Sea, 28	14	Heterozygous inv4
Fort Fordyce, 28	14	Heterozygous inv 6 and 7
Sandiles's Rest, 30	15	No rearrangements
Mbotyi, 30	15	No rearrangements

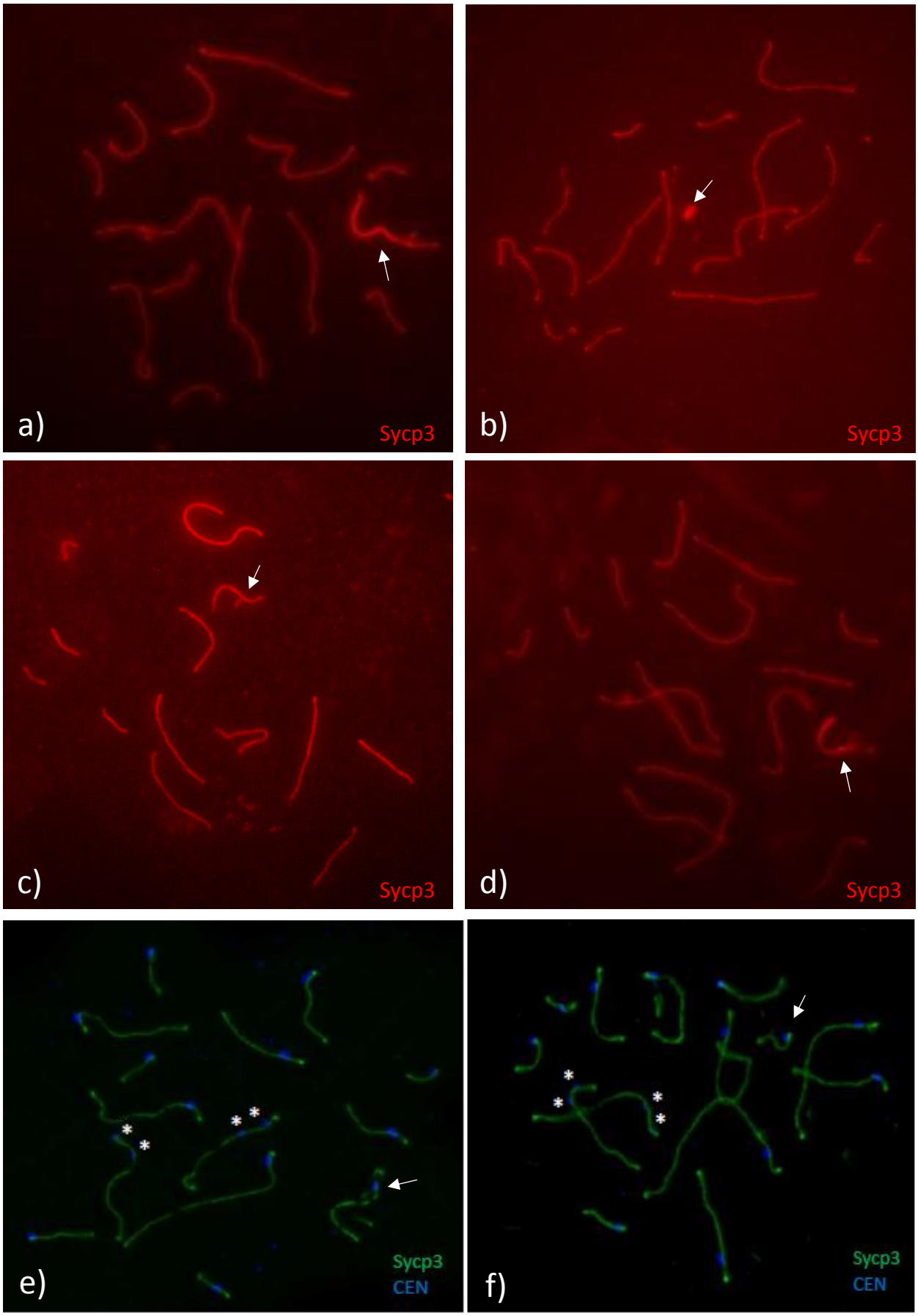


Figure 2.5. SYCP3 antibody results illustrating the lateral elements of the synaptonemal complex in 4 karyotypically variable specimens: (a) Individual from Mbotyi 2n= 30, acrocentric karyotype without rearrangements (15 bivalents), (b) Individual from Somerset-East 2n= 30, bi-armed karyotype with inversion rearrangements on OIR 4 and 6 and two B chromosomes (15 bivalents), (c) Individual from Kenton on Sea 2n= 28, bi-armed karyotype with inversion on OIR 4 (14 bivalents), (d) Individual from Kroomie with 2n= 28, bi-armed karyotype without rearrangements (standard karyotype with 14 bivalents). No inversion loops were present in individuals with inversions (b and c), homologous synapsis is present in all spreads, (e) and (f) Synaptonemal complex spreads of individual from Somerset-East, which hybridized twice with centromeric antibody CENPC on two chromosomal pairs. Arrows points to sex chromosomes and double centromeric signals indicated by*.

2.4 Discussion

2.4.1 Main findings:

This study highlights the complexity and diversity of *O. irroratus* s.l. karyotypes. Here a new inversion rearrangement on OIR7 is reported and a unique individual that has four inversions in its karyotype is documented. *Otomys irroratus* s.l. karyotypes from four localities (Kenton on Sea, Fort Fordyce, Sandile's Rest and Mbotyi) are described for the first time. Four of the previously described inversions (OIR1, 4, 6 and 10) are documented and the range of some are extended to new localities (Table 2.2, Figure 2.1). The Rb fusion chromosome OIR1/6 is also documented in a new locality, extending the range of the 2n= 24 cytoptype, where it was previously only documented in Hogsback, Kamberg and Vergelegen (Contrafatto *et al.* 1992b, Rambau *et al.* 2001). The comparisons of synaptonemal complex structure among karyotypically variable specimens revealed no clear differences in synaptic configurations and no inversion loops were seen in inversion heterozygotes. These findings cast serious doubt into whether the rearrangements previously documented in *O. irroratus* are indeed inversions and raises the possibility they represent centromeric shifts (Rocchi *et al.* 2012).

2.4.2 Broadscale cytotypic differences

When considering this results in context of all previous cytogenetic data for the species (Robinson and Elder 1987, Contrafatto *et al.* 1992a, b, Rambau *et al.* 2001, Taylor *et al.* 2009a, Engelbrecht *et al.* 2011b) certain patterns become evident. Firstly, the data confirms the presence of two major cytotypic groupings. The first group is made up of Western and Eastern Cape samples and have karyotypes that are comprised of a suite of varying bi-armed chromosomes (OIR4, 5, 6, 7, 8 and 10 sometimes acrocentric). It is also apparent that the first group is characterised by centromeric shifts or inversions on OIR1, 4, 6, 7 and 10. These

inversions are absent in the second group. In addition to the inversion rearrangements, a Rb fusion involving OIR2 and 5 has also been found in a specimen from an Alice population, which also belongs to cytogenetic group 1 (Rambau *et al.* 2001). Since these rearrangements have not been found in the other cytogenetic group it is possible that they could be synapomorphic characters. The second group which includes specimens from the north-eastern regions of the Eastern Cape, Kwazulu- Natal, Free State and Gauteng, has acrocentric karyotypes. In this group the Rb fusion 1/6, the previously described tandem fusion, as well as the acrocentric chromosomal state may be synapomorpies, as they have not been recorded outside this group. With regards to the diploid numbers, it appears that the 2n= 28 is a symplesiomorphic character, as it is present in both groups and must therefore be the ancestral state. The 2n= 24 may then conversely be considered a synapomorphy as it is only documented in the second cytogenetic group. It is however important to note that this is just a baseline 2n for each group and significant variation in this 2n is displayed throughout the ranges of each these respective clades. For example, four out of the five specimens that fit into the second group karyotypically (characterised by the 2n= 24), displayed a 2n= 30 while the remaining specimen from Mbotyi, had a 2n= 24. The two cytogenetic groups found in this investigation corresponds to the two cytotypic blueprints described by Rambau *et al.* (2001) and ultimately represents the two sister species, *O. irroratus* s.s. (bi-armed group) and *O. auratus* (acrocentric group).

2.4.3 Range expansions and new chromosome rearrangements

Four of the pericentric inversions documented previously (Engelbrecht *et al.* 2011b), a Rb fusion and a set of B chromosomes were found in the karyotypes analysed in this investigation. The tandem fusion from Hogsback and the Rb fusion OIR2/5 from Alice (Rambau *et al.* 2001) were the only rearrangements not found in this investigation. By

increasing sampling effort, the range of inversions OIR1, 6, 10 as well as Rb fusion OIR1/6 has been extended to new localities (Table 2.2, Figure 2.3, see Engelbrecht *et al.* 2011b). A novel chromosome rearrangement was detected on OIR7 and the centromeric position on this chromosome suggests it underwent a pericentric inversion or a centromeric shift (Figure 2.3). This rearrangement was found in three populations (Stellenbosch, Kroomie and Fort Fordyce) in the heterozygous state.

The specimen with the Rb fusion 1/6 interestingly had a 2n= 24 unlike the other specimen found in this locality which had a 2n= 30. The Robertsonian fusion was previously documented in an individual from Hogsback, where the fusion between OIR 1 and 6 was wrongly described as a fusion between 1 and 3 (Contraffato *et al.*1992a). The authors claim that the number of chromosomal pairs OIR8 and 9, can vary from 0-3 copies, causing further variation in 2n (Contrafatto *et al.* 1992b). These additional chromosomes were referred to as B-chromosomes, as they are analogous to the other B chromosomes found in the species complex. Considering this, and the karyotype of the specimen it is likely that the 2n= 24 observed in the specimen is due to the absence of chromosomal pair 8 and one homologue of pair 9 while the presence of this fusion in heterozygous state further explain a reduction of the 2n by one chromosome. The 2n= 30 for the remaining Mbotyi specimen and the Sandile's Rest samples could conversely be explained by additional chromosomal pairs 8. Nevertheless, this specimen represents a range expansion of 2n= 24 cytotype, as it has only been previously recorded in Hogsback, Kamberg and Vergelegen (Contrafatto *et al.* 1992b).

2.4.4 Fixation of rearrangements

The array of inversions and Robertsonian fusions documented in *O. irroratus*, begs the question as to how they became fixed in the population in the first place, considering that these rearrangements are normally negatively heterotic (King 1993). This is one of the major

challenges presented to the hybrid dysfunction theory of chromosomal speciation, as deleterious rearrangements would be selected against, hence not be able to get fixed in a population (Faria and Navarro 2010, Brown and O'Neill 2010). Central to this is whether these rearrangements occurring in *O. irroratus* s.l. are indeed underdominant (present fitness costs as heterozygotes- Rieseberg 2001). If not, fixation of the rearrangements would be easily achievable. Assuming these rearrangements are underdominant, as normally demonstrated for these types of rearrangements (King 1993), theory suggests that the rearrangements can become fixed through meiotic drive (White 1978b, Templeton 1981), drift (Spirito 1998), epistatic interactions with genes in other chromosomes (Ortíz-Barrientos *et al.* 2016) or lastly if there is a selective advantage for the genes captured within these rearrangements (Kirkpatrick and Barton 2006, Faria and Navarro 2010). The data presented here are not sufficient to conclude which of the above modes caused the rearrangements to become fixed, however it is likely that the rearrangements got fixed through homozygote advantage, due to an adaptive advantage in this state (Kirkpatrick and Barret 2015). This follows the fact that the first cytogenetic group has a restricted geographic range (Cape Fynbos – *O.irroratus* s.s.), which differs climatically from the second group (Grasslands – *O.auratus;* Engelbrecht *et al.* 2011b). Bottleneck effects and genetic drift may also have played a major part in the fixation of these rearrangements (Rieseberg 2001).

2.4.5 Stasipatric speciation in *Otomys irroratus* s.l

Preliminary molecular analysis has revealed that the acrocentric group is older and likely gave rise to the younger bi-armed group in a stasipatric pattern of speciation (Key 1968) such as observed in mosquitoes (White 1978b), grasshoppers (White *et al.* 1967, Kawakami *et al.* 2009,2011), rock wallabies (Sharman *et al.* 1989) and other species listed in King (1993). To simplify, the stasipatric speciation model suggests that contiguous or connected population

groups could diverge through the evolution of chromosomal differences. In the case of *O. irroratus* s.l. this new bi-armed cytotype likely spread from its origin point (considering *O. auratus* group is older – Engelbrecht *et al.* 2011a) in the north-eastern regions of the Eastern Cape and KwaZulu-Natal region south-westwards to the cape and in the process the hybrid zone in the Alice area originated. The species then spread into the new areas of the greater Cape, characterised by Thicket and then Fynbos bioclimatic zones which differs in vegetation type, rainfall, humidity, fire regime (amongst others) from its ancestral range encompassed by the Grassland biome (Mucina and Rutherford 2006). It is likely that the inversions evolved at this stage for adaptive purposes (Kirkpatrick and Barton 2006, Hoffmann and Rieseberg 2008) and probably became fixed through homozygote advantage or meiotic drive. In this instance the abovementioned bioclimatic regions were already established (4 – 5 mya) before *O. irroratus* s.l. underwent its south-westwards range expansion into the Cape and gave rise to *O. irroratus* s.s. (~1.3 mya- Engelbrecht *et al.* 2011a, Mucina and Rutherford 2006).

2.4.6 Persistence and identity of rearrangements; inferences from meiotic data

The stasipatric model of chromosomal speciation discussed above is based on the premise that homozygous forms of the inversions have a selective advantage. However, this does not explain the high levels of inversion heterozygotes documented in *O. irroratus*. King (1993) summarizes four mechanisms as to how these detrimental meiotic effects of inversions can be overridden: (i) the location of chiasma may be changed to a terminal position, or occur outside the inversion loop in the pachytene stage of meiosis (Supressed Recombination model); (ii) non homologous pairing of the inverted segment of a heterozygote may take place during pachytene; (iii) the association of telomeric and centromeric ends followed by the formation synapsis as reverse chromosome pairing takes place in inversion heterozygotes; and (iv) the formation of an inversion loop in the synaptonemal complex of heterozygotes that facilitate the

alignment of homologous regions. Several of these mechanisms have been documented previously e.g. chiasmata repositioning in house mice (Bidau *et al.* 2001), heterosynapsis (non-homologous pairing) in inverted regions of sand rat and deer mouse (Moses *et al.* 1982, Hale 1986) and more (Noor *et al.* 2001, Hoffman and Rieseberg 2008, Ostberg *et al.* 2013, Capilla *et al*. 2016). The data gathered in this investigation is not sufficient to conclude which of these mechanisms are operating to allow these chromosomal rearrangements to persist. I speculate that it could possibly be recombination suppression (mechanism i) that is responsible for the maintenance of inversions heterozygotes in *O. irroratus* populations. If the inversions indeed act as recombination suppressors, it would have facilitated chromosomal speciation through the accumulation of incompatibilities (Navarro and Barton 2003, Faria and Navarro 2010, Brown and O'Neill 2010). The possibility of the remaining mechanisms to be at work may be tentatively rejected, since homologous synapsis (bivalents) was observed across the board, even in specimens with inversions and no inversion loops were visible (Figure 2.5). Similar studies that compared the effects of simple and complex Robertsonian fusions on synaptonemal complex structure in shrews, also found that only the most complex chains of Rb fusions were sufficient to cause pairing irregularities in meiosis (Matveevsky *et al.* 2012, Belonogova *et al.* 2018). It is possible that effects of these inversions are not strong enough to hamper pairing and synapsis of *O. irroratus* chromosomes during meiosis.

Alternatively, it is important to note that all these so-called inversions may very well be centromeric shifts. These are very difficult to rule out on grounds of G-bands alone, as pericentric inversions rearrange the gene order hence moving the centromere, whereas centromeric shifts retains gene order, but repositions the centromere. Inversions are also well documented for the fitness consequences they hold for the carrier (Kirkpatrick 2010), whereas centromeric shifts does not alter the gene content, and consequently they are less deleterious.

There are however studies with pieces of evidence that points to the contrary; that centromeric shifts are of evolutionary significance due to genetic decay at the old and new centromeric position (Shubert and Lysak 2011; Rocchi *et al*. 2012). Evolutionary new centromeres (ENC's) have been documented in primates (Ventura *et al.* 2007), *Equus* species such as donkeys and zebras (Piras *et al.* 2010) amongst others (see Rocchi *et al.* 2012). Initially it was thought that these neocentromeres were strictly associated with satellite DNA (Piras *et al.* 2010), until Wade *et al.* (2009) found that satellite DNA were completely absent from the functional ENC on horse chromosome 11. This result proved that satellite DNA is not pre-requisite for the formation of new centromeres but will eventually be acquired as the new centromere matures (Amor and Choo 2002, Piras *et al.* 2010). According to Nergadze et al. (2018), centromere position is thus not sequence dependent, but rather governed by epigenetic factors that influences CENP binding positions.

Two lines of evidence from the synaptonemal complex data presented here, suggest that the chromosomal rearrangements found in *O. irroratus* may be centromeric shifts rather than inversions. The first is the fact that no inversion loops were found in specimens that appeared to have inversions in their G-banded karyotypes. Inversion loops are invariably associated with inversions, as it is a mechanism that assures proper pairing through synaptic adjustment (Moses *et al.* 1982, Bojko 1990, Dobigny *et al.* 2017). The absence of inversion loops could thus point to the absence of inversions. It is however essential to note that the lack of visible inversion loops may be due to effective synaptic adjustment, which then supresses loop formation through heterologous synapsis (Dobigny *et al.* 2017). Another result that suggests centromeric shifts comes from the detection of two CENPC signals, on two bivalents of a specimen, which according to the G-band data, has inversions. Epigenetic mechanisms likely caused the CENP binding site of this chromosome to undergo a short-range shift, a

phenomenon known as centromere sliding, which likely gave rise to the additional epiallele (CENPC signal -Figure 2.5) seen here (Purgato *et al.* 2015, Guilotto *et al*. 2017, Nergadze *et al.* 2018). It is quite possible that this new epiallele lacks satellite DNA and represents the early stage of a centromeric shift (Piras *et al.* 2010), resulting in a neodicentric chromosome which has an inactive and a newly activated centromeric site (Marshall *et al*. 2008, Wade *et al.* 2009). CENP proteins were previously documented in both active and inactive centromeres of dicentric mouse chromosomes (Earnshaw *et al.* 1989; Perez-Castro *et al.* 1998). Subsequent studies observed that centromere location remains stable during mitotic proliferation of cultured cells (Hori *et al.* 2017, Nergadze *et al.* 2018), explaining why no dicentric chromosomes were present in the G-banded karyotype of this specimen, which was established from fibroblast cultures. Nergadze *et al.* (2018) speculates that centromere sliding takes place when the CENP proteins are most prone to epigenetic changes and modifications, such as during meiotic division and other early developmental stages.

The 2n variation documented in *O. irroratus* is only to a small extent driven by fusion rearrangements, and more to the presence and absence of supernumery chromosomal pairs. Other rodents such as the house mouse (Piálek *et al.* 2005, Franchini *et al.* 2016) and the shrew (Wójcik *et al.* 2003, Belonogova *et al.* 2018) display 2n variation due to Robertsonian rearrangements. This creates a mosaic of chromosomal races across the distribution range of these respective species, with various combinations of metacentric and acrocentric chromosomal arms (Piálek *et al.* 2005). Introgression would reinforce these chromosomal races by restricting geneflow and in an ongoing speciation process lead to the formation of subspecies (Capanna and Castiglia 2004). *Otomys irroratus* is different as the rearrangements in its karyotypes represent a suite of floating polymorphisms, including heterozygous

individuals, which co-exists within a population outside a contact zone. This ultimately makes *O. irroratus* the ideal model candidate for studies of chromosomal speciation.

2.4.7 Concluding remarks

The inversion rearrangements OIR1, 4, 6, 7 and 10 as well as the Rb 2/5 could act as synapopmorphic characters defining the bi-armed cytogenetic group (*O. irroratus*), as it is absent in the second group. While the Rb fusion 1/6, tandem fusion as well as the acrocentric chromosomal state can be considered a synapopmorphies characterising the second cytogenetic group (acrocentric group -*O. auratus*). Here we also found potential evidence for centromeric shifts and present three putative scenarios: i) rearrangements are not inversions but are all centromeric shifts, hence no inversion loops; ii) rearrangements are inversions, but inversion loops suppressed through synaptic adjustment; or iii) rearrangements are a combination of inversions and centromeric shifts. BAC clones developed and linked to the chromosome map of the house mouse (MMU- Korenberg *et al.* 1999) could be used to unequivocally validate the identity of the rearrangements. Regardless which scenario holds, this study ultimately suggests that these rearrangements are not deleterious (based on IF data) and hence does not constitute effective post zygotic isolation mechanisms. This will then explain how these rearrangements prevail and persist in a population of *O. irroratus* e.g. individual with 4 inversions. Further optimization and application of the MLH1 antibody would be beneficial to quantitatively assess whether recombination is indeed supressed in inverted and rearranged chromosomes. It would also be interesting to re-examine the supposed evolutionary new centromere revealed by CENPC immunofluorescence, with single nucleotide polymorphisms (SNP's) or ChIP-sequencing to explore whether the ENC is underpinned by satellite DNA.

Chapter 3: Link between chromosomal inversions and mitochondrial divergence in *Otomys irroratus*

3.1 Introduction

The vlei rat *O. irroratus* s.l. is endemic to the African continent, with a distribution that encompasses the mesic parts of South Africa, as well as certain parts of Swaziland, Zimbabwe and Mozambique (Skinner and Chimbamba 2005, Monadjem *et al.* 2015). Before allozymes (Taylor *et al.* 1992, Contrafatto *et al.* 1997), chromosomes (Robinson and Elder 1987, Contrafatto *et al.* 1992a, b, Engelbrecht *et al.* 2011b), mtDNA (Maree 2002, Engelbrecht *et al.* 2011a) as well as cranial morphometric approaches have been used to examine the population structuring of this species complex (Taylor *et al.* 2004, 2009a). Allozymes revealed little intraspecific variation, and high levels of gene flow between populations, indicative of a panmictic genetic structure for *O.irroratus* s.l. (Taylor *et al.* 1992).

Mitochondrial cyt *b* sequences revealed the presence of two genetic lineages, demarcated by a sequence divergence of 6.4 % (Taylor *et al.* 2009a). These two lineages also coincide with the two main cytotypic blueprints described for the species (Rambau *et al.* 2001). In agreement with the divergent chromosomal and mitochondrial lineages, Pillay *et al.* (1992, 1995) showed a breakdown of mate recognition cues, increased aggression and reduced reproductive success when conducting interpopulation crosses between specimens with the tandem chromosome (cytotype A1) and without (cytotype A2). Ultimately this proved the presence of both pre-mating and postzygotic reproductive isolating mechanisms within *O. irroratus* s.l. population groups (Pillay *et al.* 1992, 1995). Divergence within *O. irroratus* s.l. was further substantiated when the analyses of craniometric characters revealed two main morphological groupings according to climate (Taylor *et al.* 2009a). The first grouping consisted of specimens from the Fynbos and Albany thicket biome (*O.irroratus*), while the

second grouping recognized specimens in the Grassland biome (*O. auratus*-Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a, Monadjem *et al.* 2015).

The evolutionary history of *O. irroratus* s.l. as well as several other small mammals in South Africa, was shaped by climatic oscillations since the Pliocene (Matthee and Robinson 1997, Smit *et al.* 2007, Edwards *et al.* 2011). Climatic fluctuations taking place across a topographically varied landscape (e.g. rivers, mountains and forests) would ultimately generate isolated habitat patches (Montgelard and Matthee 2012), and present major challenges, especially if the animal is not a habitat generalist such as *O. irroratus* s.l. *Otomys irroratus* s.l occurs in mesic grasslands (Skinner and Chimimba 2005), which are believed to have fragmented due to the expansion and contraction of forests in South Africa, since the Last Glacial Maximum (Lawes *et al.* 2007). Although historical and contemporary anthropogenic activity would also play a role in transforming and fragmenting favourable habitats (Lawes *et al.* 2000, Mbora *et al.* 2009), climatic oscillations were instrumental in shaping the population structure of several mammal species (Montgelard and Matthee 2012). Small mammals such as *O. irroratus* s.l. have limited dispersal abilities (Lidicker 1975) which facilitates allopatric divergence as migration and gene flow is hampered. Isolated populations would eventually become locally adapted and genetically divergent from other population groups.

Pericentric inversions are widely present in *O. irroratus* karyotypes. These inversions, particularly in heterozygous state are well known for the deleterious consequences it holds for the carrier due to irregular pairing during meiosis and the subsequent formation of aneuploid gametes (King 1993, Kirkpatrick 2010). Pericentric inversions could in this way drive the divergence of species (Noor *et al.* 2001). Inversions can also facilitate the divergence of species through adaptive purposes. By modifying patterns of recombination, inversion may create favourable allelic combinations that confer an adaptive advantage to new or specific

environmental pressures (Kirkpatrick and Barton 2006, Hoffman and Rieseberg 2008, Ullastres *et al.* 2014). Here I investigated whether there is a correlation between mtDNA divergence and inversion rearrangements.

The barcoding gene, the mitochondrial cytochrome *c* oxidase subunit 1 (COI) has recently became the go-to gene for species identification (Herbert *et al.* 2003, Waugh 2007, Cai *et al.* 2011). Whereas most mammalian phylogenies/phylogeographies are based on the cyt *b* gene (Rambau *et al* 2003, Willows-Munro and Matthee 2009, Russo *et al.* 2010, Demos *et al.* 2014), the number of studies exploring the barcodes of small mammals specifically rodents, are limited (Clare *et al.* 2007, Borisenko *et al.* 2008, Nesi *et al.* 2011). Consequently, mammalian taxa are still fairly underrepresented in the Barcode of life database (http://www.barcodeoflife.org/ - Herbert *et al.* 2003). Using both mtDNA markers, COI and cyt *b*, would not only add to the limited mammalian barcodes but would also allow the opportunity to test the utility of COI in resolving phylogeographies.

The aims of this study were:

1 To refine mtDNA relationships between various chromosomal populations of *O. irroratus* s.l. and to determine whether there is a correlation between mtDNA divergence and chromosome rearrangements (specifically inversions rearrangements), by comparing mtDNA divergence values (uncorrected p –distances) of specimens with and without these rearrangements.

2 To test the utility of the barcoding gene COI against the cyt *b* gene, in resolving population genetic structure of this species

3.2 Materials and Methods

3.2.1 Sample and locality information

Thirty-four specimens of *O. irroratus* were collected in the Western and Eastern Cape provinces of South Africa. Tissue for an additional 60 samples, from 13 different localities, was kindly provided by collaborators (Table 3.1) resulting in a total of 94 specimens with fresh tissue for DNA extractions.

3.2.2 DNA sequencing and amplification

Tissue samples for DNA extraction were stored in 96% ethanol. Total genomic DNA was extracted following the manufacturer protocol of a Macharey-Nagel™ extraction kit. In short, this involved Proteinase K digestion, after which the DNA solution was put through a set of purification washes before it was stored at 4°C. Most of the COI gene as well as the complete cyt *b* genes were amplified using universal primers, L 14724 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3') (Kocher *et al.* 1989), H 15915 (5'–CTG CAG TCA TCT CCG GTT TAC AAG AC-3') (Irwin *et al.* 1991) and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO 2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.* 1994). Each PCR reaction consisted of 23.8 µl of millipore water, 5 µl of 25mM $MgCl_2$, 5 µl of 10 x Mg^{2+} free buffer, 4 µl of 10 mM dNTP's, 5 µl of one the respective primer pairs at 10 mM, 0.2 µl *Taq* polymerase and 2 µl of the template DNA. PCR reactions were taken through temperature cycles of 94°C for 4 min, 94°C for 30 seconds, 47°C for 45 seconds and 72°C for 35 seconds. The last three steps were repeated for 35 cycles before the final extension period of 15 min at 72°C. For COI amplifications the annealing temperature was reduced from 47°C to 45°C. To test for successful amplification, DNA products were run out on a 1% agarose gel, for 1 hour at 100 V. Successful amplification

products were gel purified with a biospin gel extraction kit (BioFLux™), following manufacturers' protocol. Briefly, this involved melting the DNA containing gels in extraction buffer, a set of washes in buffered solutions then a final elution stage which would contain the purified PCR product. Sequencing was done at the Central Analytical Facility at the University of Stellenbosch using the Big Dye chemistry.

3.2.3 Molecular analysis

Raw sequence files were edited and aligned using BioEdit sequence alignment editor ver. 7.2.5 (Hall 1999). ModelTest implemented in MEGA 7 (Kumar *et al.* 2016) was used to identify the substitution model which best fit the sequence data of the cyt *b* and COI genes. Phylogenetic relationships were then inferred for each of the datasets using the Neighbour joining (NJ) and Maximum parsimony algorithms (MP) as implemented in and MEGA 7 (Kumar *et al.* 2016) and PAUP*4 ver. 10 (Swofford 2002). Bootstrapping (Felsenstein 1985) was used to assess confidence in the nodes (> 75% considered as well supported, while those <75% were considered poorly supported). *Otomys laminatus* (FJ619558) and *O. karoensis* (FJ619560) were used as outgroups as they are sister taxa to *O. irroratus* (Taylor *et al.* 2009b, Engelbrecht *et al.* 2011b).

Sequence divergence values were generated from uncorrected p-distances in MEGA 7. Population level analysis were done in DnaSp ver 4.10.9 (Rozas *et al.* 2003) to determine the number of haplotypes, haplotype diversity (*h*), nucleotide diversity (π) and the pairwise nucleotide diversity (*k*) among different molecular taxonomic units. A haplotype network was drawn using the median joining criteria of Network ver.5.0.0.3 (Bandelt *et al.* 1999). The population demographics of *O. irroratus* were evaluated using mismatch analysis, to determine whether a range expansion or contraction has taken place historically. Mismatch distributions

of pairwise nucleotide differences for each group were calculated and compared with models for expanding/contracting populations (Rogers and Harpending 1992).

Table 3.1: List of specimens used for phylogenetic inference. Locality name and province where found, the number of specimens from the given locality and as well as the GPS coordinates where given specimens were sampled, are shown. The cyt *b* sequences was supplemented with sequences from other studies and are represented by Genbank accession numbers in the last column (Maree 2002, Taylor *et al.* 2009, Engelbrecht *et al.* 2011b).

	Locality/province (Number of specimens)	Coordinates	Cyt *b* or COI	Fresh tissue/ Genbank accession nr
		Western Cape		
1.	Stellenbosch (N=21)	33°55'49''S, 18°49'44''E 33°55'54''S, 18°49'47''E	Both	This study, HM363669- HM363672
2.	Bainskloof (N=8)	33°34'50''S, 19°09'10''E	Cyt *b* only	FJ619538, FJ619548, FJ619549, AF492718, AF492721, HM363696, HM363697, HM363711
3.	Porterville (N=5)	32°59'15''S, 19°01'28''E	Both	This study, HM363678- HM363682
4.	Algeria (N=4)	32°26'19''S, 19°05'05''E	Cyt *b* only	FJ619546, FJ619547, AF492720, HM363710,
5.	Swartberg Mts (N=1)	33°21'36''S, 22°03'14''E	Cyt *b* only	FJ619539
6.	De Hoop (N=5)	34°26'03''S, 20°32'52''E	Both	This study, HM363693
7.	Oudtshoorn (N=1)	33°39'56''S, 22°07'39''E	Cyt *b* only	HM363694
8.	Buffelsjagsrivier (N=2)	34°04'31''S, 20°53'36''E	Both	This study
9.	Beaufort West (N=4)	32°15'19''S, 22°34'25''E	Cyt *b* only	HM363685-HM363688
10.	Gamkaskloof (N=1)	33°21'50''S, 22°03'41''E	Cyt *b* only	HM363713
		Eastern Cape		
11.	Baviaanskloof (N=5)	33°39'38''S, 24°37'57''E	Both	This study, HM363664-HM363668
12.	Somerset-East (N=11)	32°42'75''S, 25°38'55''E 32°41'62''S, 25°37'80''E	Both	This study, HM363689 - HM363692
13.	Kroomie (N=14)	32°45'76''S, 26°23'63''E 32°47'19''S, 26°28'21''E 27°08'07''S, 20°32'11''E	Both	This study, HM363673 - HM363677
14.	Alice (N=6)	32°47'00''S, 26°50'12''E	Both	This study, HM363698, AH012645, AF492717
15.	Groendal NR (N=5)	33°42'07''S, 25°19'20''E	Cyt *b* only	FJ619536, FJ619537, AF492719, HM363699 HM363700,
16.	Fort Beaufort (N=3)	32°43'21''S, 26°38'03''E	Both	This study
17.	Hogsback (N=13)	32°36'19''S, 27°01'30''E	Both	This study, FJ619553, AF492714, HM363657-HM363663 , HM363708,
18.	Mbotyi (N=2)	31°25'22''S 29°43'47'' E	Both	This study

19.	Sam Knott NR (N=3)	33°05'27''S, 26°42'58''E	Cyt *b* only	FJ619543, HM363718, HM363719, FJ619541
20.	Kenton on Sea (N=4)	33°34'05''S, 26°34'25''E	Both	This study
21.	Fort Fordyce (N=3)	32°40'86''S, 26°28'94'' E 32°39'96''S, 26°29'37'' E	Both	This study
22.	Grahamstown (N=6)	33°19'19''S, 26°31'04''E 33°18'15''S, 26°31'08''E	Both	This study, HM363701, HM363702, HM363714-HM363716, FJ619540
23.	Sandile's Rest (N=1)	32°40'06''S 27°17'27''E	Both	This study
		Kwazulu-Natal		
24.	Lemonwood Forrest (N=3)	29°28'03''S, 30°06'16''E	Cyt *b* only	HM363704- HM363706
25.	Tygerskloof (N=2)	25°58'05''S, 31°34'01''E	Cyt *b* only	FJ619554, FJ619555
26.	Dargle (N=2)	29°50'20''S, 30°03'30''E	Cyt *b only*	FJ619550, FJ619551
27.	Kamberg (N=3)	29°24'54''S, 29°40'21''E	Cyt *b* only	FJ619552, AF492715, HM363707
28.	Karkloof (N=3)	29°18'18''S, 30°13'30''E	Cyt *b* only	AF492716, HM363709, FJ619557
		Free State		
29.	Bloemfontein (N=1)	29°07'02''S, 26°14'01''E	Cyt *b* only	HM363656
30.	Ficksburg (N=1)	28°47′33''S, 27°53′35''E	COI only	This study
31.	Theunissen (N=2)	28°30'06''S, 26°48'07''E	Cyt *b* only	HM363683, HM363684
		Gauteng		
32.	Municipal Springs (N=1)	26°21′07''S, 28°45′00''E	Cyt *b* only	FJ619556
		Zimbabwe		
33.	Chingamwe (N=1)	18°45′00''S, 32°57′00″E	Cyt *b* only	FJ619562

3.3 Results

In total a 1137 bp fragment of the cyt *b* gene was successfully sequenced for 47 samples, and a 660 bp fragment of the COI gene was successfully sequenced for 89 samples. The cyt *b* data was supplemented with sequences from Genbank, N=129 (Table 3.1).

3.3.1 Phylogenetic analysis

The HKY + G and TN92 + G substitution models were selected by MEGA7 for cyt *b*, and COI datasets respectively based on BIC (Bayesian Information Criterion) (Hasegawa *et al.* 1995, Nei and Kumar 2000, Kumar *et al.* 2016). These models retrieved the lowest BIC score and best described the substitution patterns at hand. The NJ tree topologies retrieved from the cyt *b*, COI and combined mitochondrial markers were generally congruent (Figure 3.1 – 3.3), with minor differences in the bootstrap values (Table 3.2). Two monophyletic clades were retrieved in all analysis. The mean sequence divergence between clade 1 and 2 was 5.6% and 2% with cyt *b* and the combined datasets respectively and 4.5 % with the COI dataset. The first large clade (clade 1) was well supported (>75% bootstrap) by all three datasets and consisted of samples of the Western Cape (Stellenbosch, Bainskloof, Porterville Algeria, Swartberg Mts, De Hoop, Oudsthoorn, Buffelsjagsrivier, Beaufort West and Gamkaskloof) and Eastern Cape provinces (Baviaanksloof, Somerset-East, Kroomie, Alice, Groendal NR, Fort Beaufort, Sam Knott NR, Kenton on Sea, Fort Fordyce, and Grahamstown). This clade was weakly structured in all tree topologies with short branch lengths and weak nodal support, suggestive of a recent period of rapid diversification. This clade is further subdivided into two sub clades 1a and 1b- both had poor bootstrap support with all but the combined dataset (Table 3.2). Subclade 1a is comprised of the bulk of the Eastern-Cape and a portion of the Western-Cape samples whereas 1b, was small and consisted of the remaining Western-Cape samples.

The second clade consists of samples from the north-eastern regions of the Eastern Cape (Hogsback, Mbotyi, Sandile's Rest), Kwazulu-Natal (Lemonwood Forrest, Tygerskloof, Dargle, Kamberg and Karkloof), Free State (Bloemforntein, Ficksburg and Theunissen), Gauteng (Springs) and one specimen from Zimbabwe and was well supported with the all but the COI dataset (Table 3.2). This clade is also subdivided into two smaller clades 2a, which is poorly supported with the COI marker and 2b which was well supported by all three datasets. Subclade 2a consists of samples from the Free State, Kwazulu-Natal, Gauteng and Zimbabwe (Figure 3.1) and it is represented by the cyt *b* dataset where it is only well supported by the NJ cyt *b* tree and the combined dataset. Subclade 2b is well supported by all three datasets and consists of specimens from the north-eastern regions of the Eastern- Cape and Kwazulu-Natal and included specimens from Sandile's Rest, Mbotyi and Hogsback (Figure 3.1 – 3.3). Interestingly, two samples from Kroomie also grouped in this clade, where the remaining of the samples from these localities grouped in 1a. These groupings were consistent in all the tree topologies although some of these regions were not represented in the smaller COI and combined datasets. Similarly, two main clades separated by numerous mutations were retrieved from the haplotype network (Figure 3.4). The first haplotype clade had a high connectivity of haplotypes, with short distances (mutational steps) between. The second haplotype clade had lower connectivity and larger distances between indicative of higher within clade divergences.

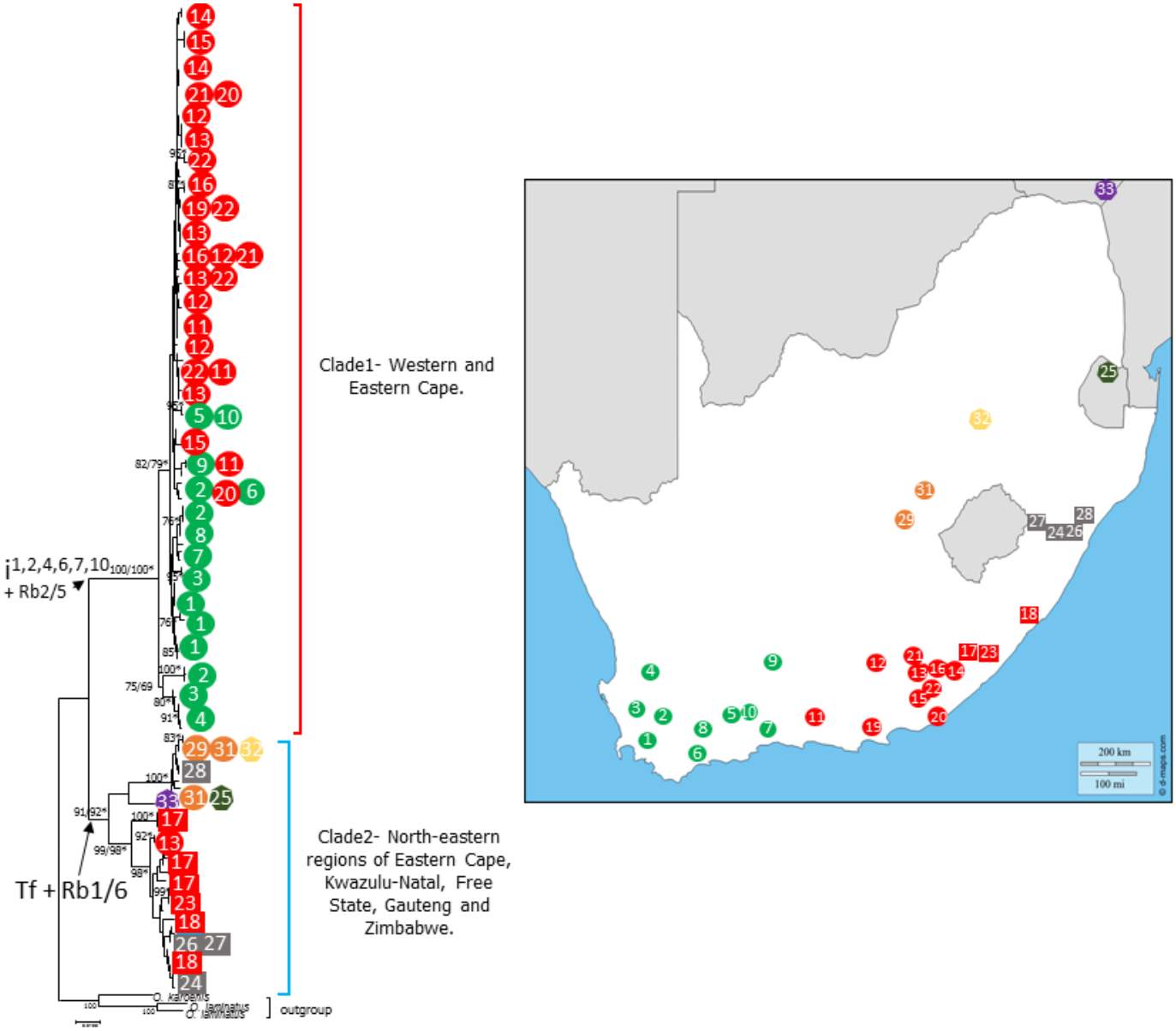


Figure 3.1. Neighbour joining tree of cyt *b* retrieved from 129 specimens 1137 base pairs, with map illustrating sequence localities. Each number on the tree corresponds to its position on the map, listed in table 3.1. Two well supported clades were retrieved: Clade 1 consists of samples from Western Cape (Green) and Eastern-Cape (Red) whereas clade 2 consists of samples from the north-eastern regions of the Eastern Cape, Kwazulu- Natal (Gray), the Free State (Orange), Gauteng (Yellow), Swaziland (Dark Green) and Zimbabwe (Purple). The chromosomal data collected in chapter 2 was added to the tree and revealed an occurrence of inversion rearrangements in clade 1 which were absent in clade 2. Clade 1 on the other hand lacks the Robertsonian fusion between OIR1 and 6, the occurrence of 2n= 24 karyotypes, and the tandem fusion which has only been documented in clade 2, to date. In general clade 1 had karyotypes that consisted of bi-armed chromosomes (Indicated by circles) whereas clade 2 had karyotypes consisting of acrocentric chromosomes (Indicated by squares). Localities with unknown karyotypic configurations indicated by a heptagon (25, 32 and 33). Two specimens from Kroomie grouped in clade 2 according to cyt *b* data but in clade 1 chromosomally. Nodal confidence was achieved through bootstrapping MP and NJ trees 1000 replicates (>75% considered well supported*), indicated for major clades only as (MP bootstrap/NJ bootstrap).

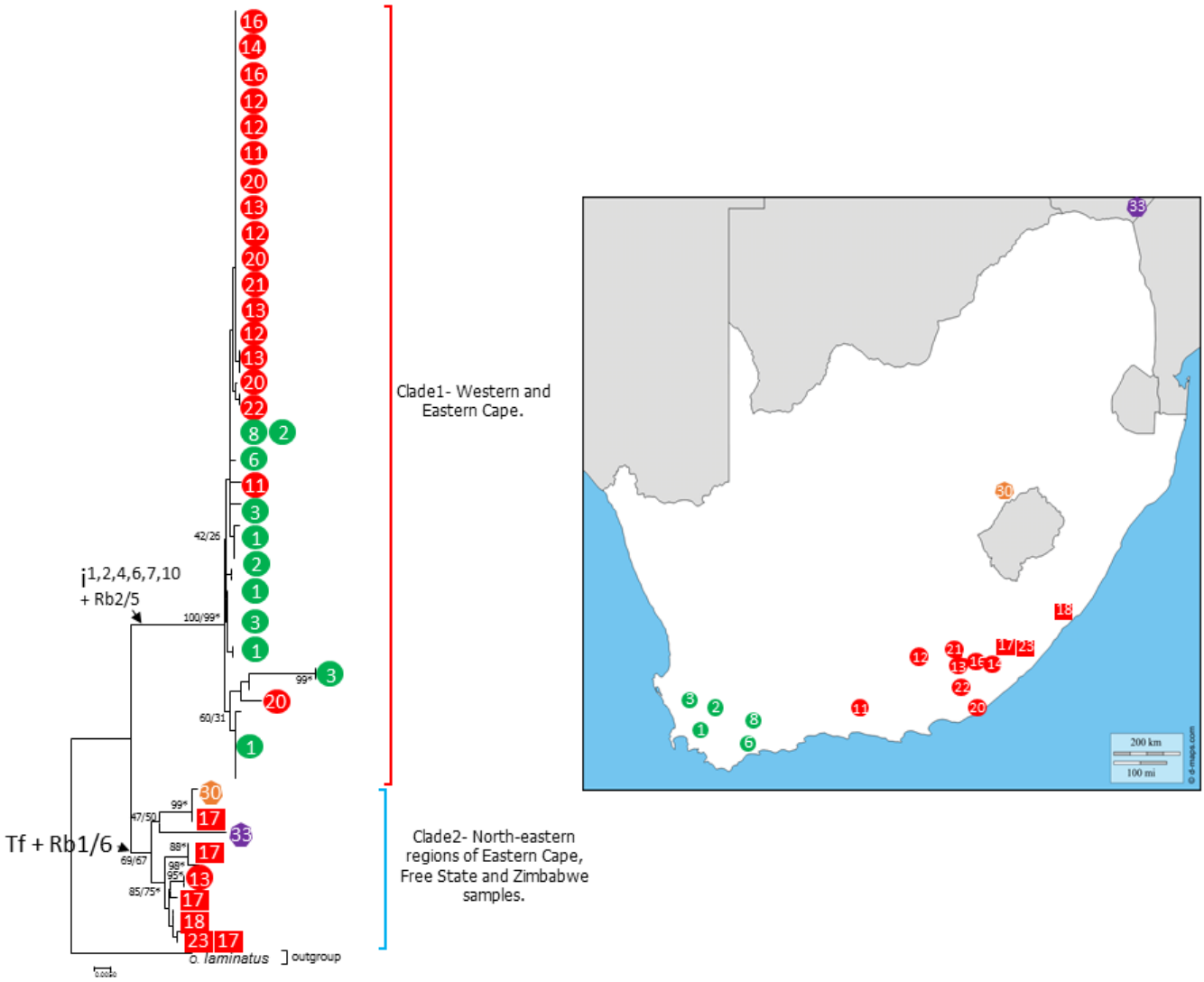


Figure 3.2. Neighbour joining tree of COI inferred from 89 specimens and 640 base pairs with partial map of South Africa, illustrating sequenced localities. The COI tree topology strongly corroborates the typology as inferred from cyt *b*, showing monophyly of *O. irroratus*. The two clades which was retrieved was identical in composition to the clades of the cyt *b* topology, however only with a reduced dataset. The two specimens from Kroomie retrieved the same unusual position on the tree as with the cyt *b* dataset. Nodal confidence was achieved through bootstrapping MP and NJ trees 1000 replicates (asterisks indicates well supported clades), indicated for major clades only as (MP bootstrap/NJ bootstrap).

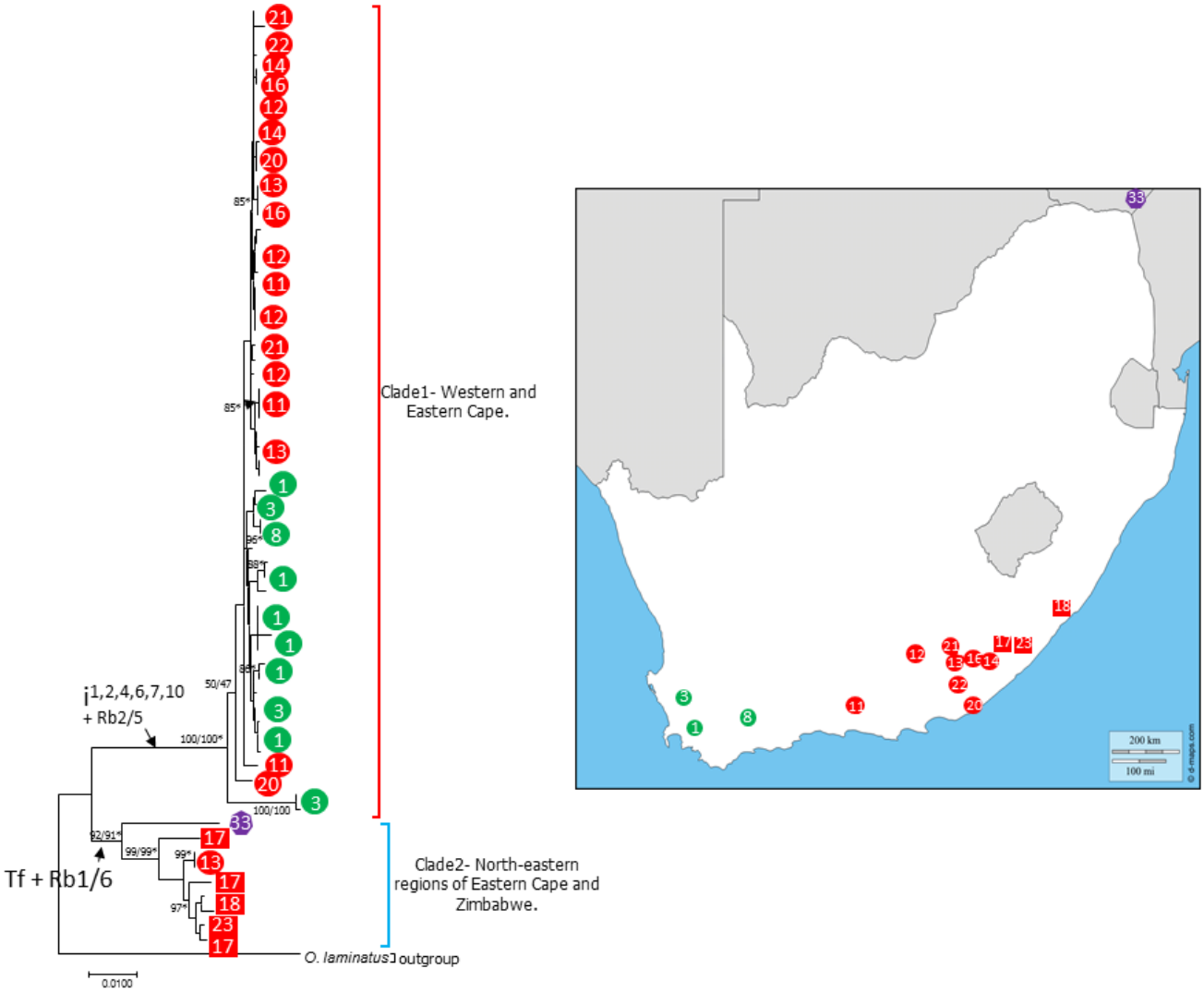
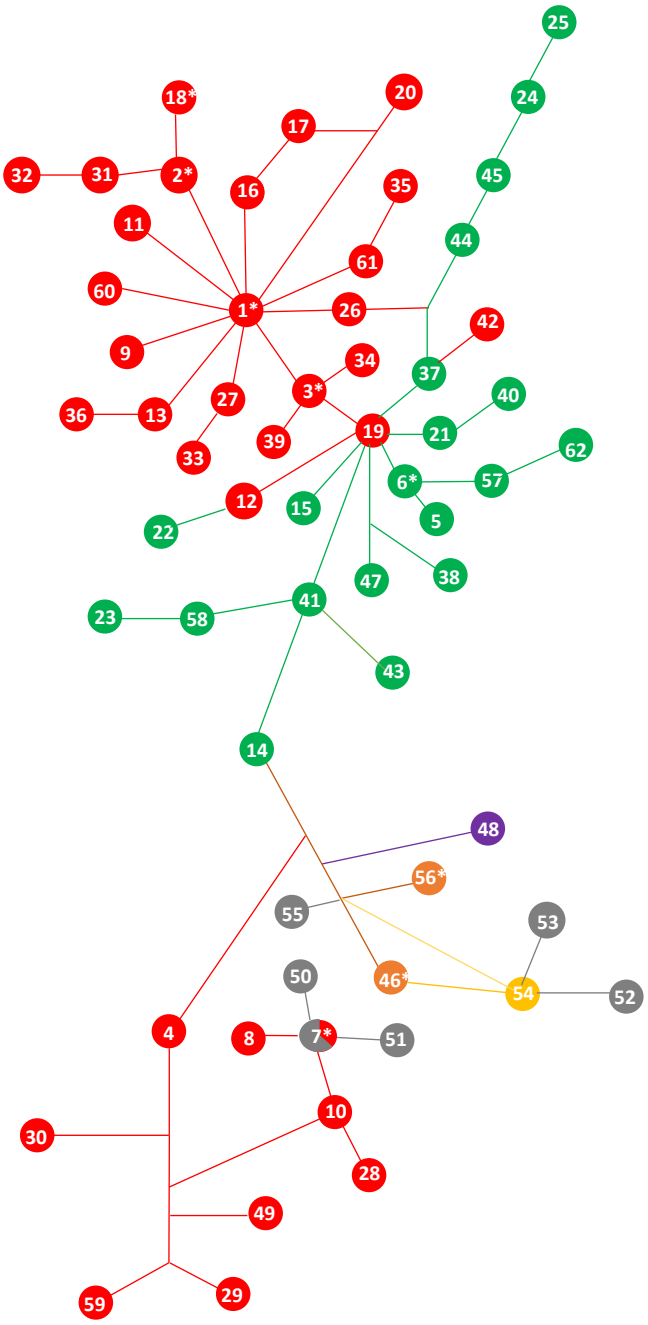


Figure 3.3. Neighbour joining tree of combined and reduced COI and cyt *b* datasets for comparison. Inferred from 66 specimens and a total of 1795 base pairs with a map of South Africa, showing approximate sequenced localities. The combined tree topology strongly corroborates the typologies of both independent datasets, supporting the monophyly of *O. irroratus.* The two clades which were retrieved were identical in composition to the clades retrieved by both COI and cyt *b* markers. Nodal confidence was achieved through bootstrapping MP and NJ trees 1000 replicates (asterisks indicates well supported clades), indicated for major clades only as (MP bootstrap/NJ bootstrap) Bootstrap values of well supported NJ nodes indicated on tree.

When the chromosomal data is plotted on the mtDNA topographies it reveals that inversions on OIR1, 2, 4, 6, 7, and 10 are restricted to clade 1. A Rb fusion involving OIR chromosome 2 and 5 also occurred on one specimen from Alice (Clade 1) in a previous study and has not been documented outside this clade. Clade 2 on the other hand is characterised by a tandem fusion chromosome and a Rb fusion involving OIR 1 and 6 which has not been documented in any populations in clade 1. The two clades also differ broadly with regards to the morphology of the chromosomes. Members of clade 1 have bi-armed chromosomes whereas clade 2 has acrocentric chromosomes. There are however exceptions to this, as the two specimens from Kroomie that retrieved an unusual position in clade 2, was revealed to have bi-armed karyotypes, as well as some specimens from populations in the Free State province.

Table 3.2: Summary of nodal support figures for major clades inferred from bootstrapping Maximum Parsimony and Neighbour Joining trees using the cyt *b*, COI and combined datasets.

Dataset	Clade 1	Clade 2	Subclade 1a	Subclade 1b	Subclade 2a	Subclade 2b
Cyt *b* (1137 bp)	100/100	91/92	82/79	75/69	67/96	99/98
COI (660 bp**)**	100/99	69/67	42/26	60/31	47/50	84/75
Combined (1795 bp)	100/100	92/91	50/47	100/100	100/100	99/99



Locality	Haplotypes
Stellenbosch	5,6*,14,15,57,62
Bainskloof	22,23,25
Porterville	6*,41,44,45
Algeria	24
Swartberg Mts	21
De Hoop	47
Oudsthoorn	43
Buffelsjagsrivier	58
Beaufort West	37,38
Gamkaskloof	40
Baviaanskloof	31,39,42
Somerset East	1*,27,33,34,
Kroomie	2*,3*,4,32
Alice	1*,16,17,61
Groendal	19,20
Fort Beaufort	2*, 60
Hogsback	28 ,29 ,30 ,49 ,59
Mbotyi	7*,8
Sam Knott	2*
Kenton on Sea	11 ,12
Fort Fordyce	1*,9
Grahamstown	2*,3*,18 ,26 ,36
Sandile's Rest	10
Lemonwood Forrest	7* ,51
Tygerskloof	52 ,55
Dargle	51, 7
Kamberg	50
Karkloof	53
Bloemfontein	46*
Theunissen	46*,56
Municipal Springs	54
Chimgamwe	48

Figure 3.4. Haplotype network spanning the 62 haplotypes retrieved from the cyt *b* dataset. Each numbered circle represents a given haplotype colour coded per province it can be found (see Figure. 3.1). Localities where haplotypes were documented are given in the legend to the right. Haplotypes shared between localities indicated by asterisk. Haplotype 1 (N= 11), Haplotype 2 (N= 7), Haplotype 3 (N= 6), Haplotypes 4, 15, 17 – 19, 22, 37, 38, 46, 58, 60 (N= 2), Haplotypes 5, 24, 35 (N= 4), Haplotype 6 (N= 5), Haplotypes 7, 11, 20, 23, 25, 28, 30, 49 – 51, 53 (N= 3), Haplotypes 8 – 10, 12 – 14, 16, 21, 26, 27, 29, 31 – 34, 36, 39 – 45, 47 – 48, 52, 54 – 57, 59, 61, 62 (N= 1). Network not drawn according to scale.

3.2.2 Population genetics within clade

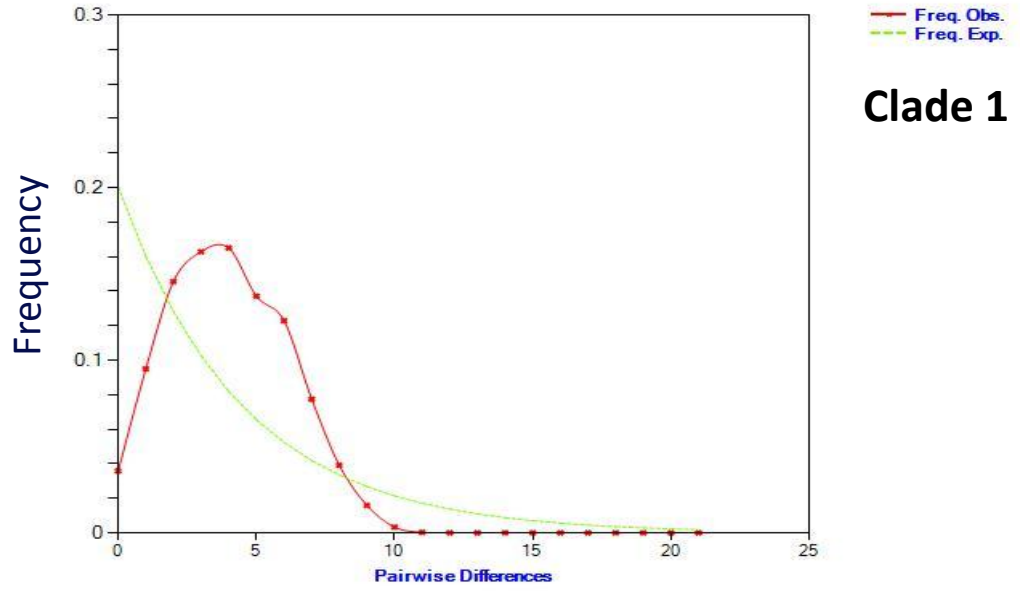
Genetic diversity statistics was calculated for each of the three datasets. For all the datasets, the haplotype diversity was high; (cyt *b:* h= 0.98 ± 0.004, COI: h= 0.86 ± 0.03, combined: h= 0.96 ± 0.014) and the nucleotide diversity was low (cyt *b:* π = 0.031 ± 0.003, COI: π = 0.021 ± 0.002, combined: π= 0.018 ± 0.003). The pairwise nucleotide differences were k = 26.862 (cyt *b*), k = 13.230 (COI) and k= 28.088 (combined). A total of 62 haplotypes were found from the 129 sequences analysed with cyt *b*, 30 haplotypes were found from the 88 sequences analysed with COI and 42 haplotypes were found from the combined dataset of 65 sequences. There were 129 polymorphic sites (115 parsimony informative) for cyt *b*, 134 polymorphic sites (67 parsimony informative) for the COI dataset and 183 polymorphic (135 parsimony informative) sites in the combined dataset.

The genetic stats were calculated separately for each of the main clades retrieved from the mtDNA sequence analyses, using the cyt *b* dataset as it is larger and more comprehensive. These results are summarized in Table 3.3. The haplotype diversities were similar for both clades however the nucleotide diversity of the second clade was significantly higher for clade 2. This is indicative of a stable population (Figure 3.5B), whereas the low nucleotide diversity of the clade 1 (Figure 3.5A) points towards a recent and ongoing diversification. These results also suggest that clade 2 is older than clade 1. Within clade sequence divergence is also much higher for clade 2 than it is for clade 1. Pairwise nucleotide difference of the entire population (clade 1 and 2) retrieved a distribution similar to that found for clade 2 (data not shown) suggesting *O. irroratus* s.l is in a stable evolutionary state overall.

Table 3.3: Genetic diversities of each of the two respective clades found with the sequencing analyses of cyt *b* (N=129).

Summary statistics	**Clade 1**	**Clade 2**
Haplotypes diversity (*h*)	0.96	0.97
#Haplotypes	44	18
Nucleotide diversity (π)	0.0047	0.0388
Pairwise nucleotide differences (*k*)	3.990	36.349
Tajima's D	-0.99	1.38
Sequence divergence	0.2 – 2.1%	2-5.0%
Chromosome rearrangements	Inversions OIR1,2,4,6,7,10, Rb fusion 2/5	Tandem fusion, Rb fusion 1/6

A)



B)

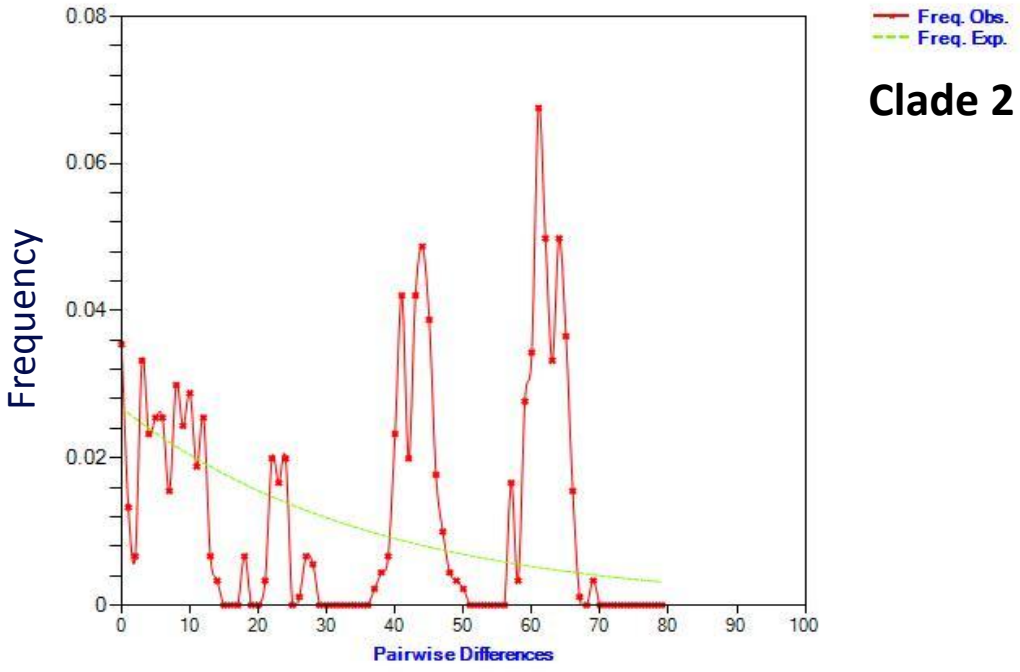


Figure 3.5. Distribution of pairwise nucleotide differences of the two clades of *O. irroratus* inferred from 129 sequences of the mtDNA cyt *b* gene. The criteria for constant population size was used to determine expected (indicated in green) and observed (indicated in red) nucleotide differences as generated in DnaSP v5. (A) Clade 1 has a unimodal distribution of the observed nucleotide differences, indicating a recently expanded population whereas (B) clade 2 and the overall population (clade 1 + 2- not shown) shows a multimodal distribution of the observed nucleotide differences, indicating a stable and older population.

3.4 Discussion

3.4.1 Main findings

The mitochondrial sequence data retrieved two large, statistically well supported clades with the combined and individual mitochondrial cyt *b* and COI markers. The phylogenies retrieved from the three datasets were congruent, however the nodal support of the COI tree was often lower than it was for the cyt *b* and combined trees (Table 3.3). The first large clade consisted of samples from the Western Cape and Eastern Cape, while the second clade consisted of samples from the north-eastern regions of the Eastern Cape, Kwazulu Natal, Free State and Gauteng. These clades are underpinned by chromosomal cytotypes and supports what has been documented in literature.

The sequence divergence between these two large clades are sufficient (~5.6% with cyt *b*), according to the divergence thresholds of Bradley and Baker (2002), to support the recognition of a cryptic sister lineage in *O. irroratus*, recently recognized as *O. auratus* (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a). These two lineages also correspond to the two main cytotypes of *O. irroratus* s.l. karyotypes (Rambau *et al.* 2001), with the first being characterised by inversion rearrangements on OIR1, 2, 4, 6, 7, 10 and Rb fusion 2/5 while the second clade is characterised by a tandem fusion chromosome as well as a Rb fusion 1/6. Additionally, these two lineages have support based on dorsal and ventral cranium size measurements too, with two morphological groups coinciding with the Fynbos and Albany thicket biome (clade 1) and the Grassland biome (clade 2) (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a). Further genetic analysis suggests that clade 1 (corresponding to *O. irroratus*) is younger or went through a more recent genetic bottleneck, whereas clade 2 (corresponding to *O. auratus*) is older and more stable. This is also evident from the low cyt *b* sequence divergence between members of clade 1 (0.2 – 2.1%) and the short branch lengths of the tree topology, compared to clade 2

which had higher sequence divergences $\bar{x}$ = 3.9% - cyt *b*) and longer branch lengths. The clade 1 samples of the haplotype network was also tightly linked with high connectivity, consistent with the evolutionary state of the clade.

The congruence found between the cyt *b* and COI tree topologies demonstrated the utility of the COI gene marker for the detection of intraspecific genetic variation in this small mammal. Although the nodal support was often weaker than the nodes retrieved for the cyt *b* and the combined trees, the nodes was identical in composition to those of the other trees. Also, the sequence divergence value between the two main clades was also slightly lower for COI (4.5%) than it was for the cyt *b* dataset (5.6%). It however important to note that these comparisons was based on a much shorter sequence length for COI (660bp) than for cyt *b* (1140bp). Regardless, this highlight a degree of accuracy for this marker, in delineating intraspecific relations within this taxon, although it may not be as useful or accurate for other mammalian taxa. When it was used in bats for instance, this marker could not discriminate between two morphologically divergent fruit bats (Nesi *et al.* 2011), whilst in other studies looking at bats and other small rodents, it could detect both intra and interspecific genetic variation (Clare *et al.* 2007, Borisenko *et al.* 2008). As the barcode database of mammals grows, the utility of this gene would become clearer.

3.4.2 Chromosomal rearrangements as driver for mtDNA divergence in *O. irroratus*?

Chromosomal rearrangements such as inversions, are often negatively heterotic as they could lead to the production of unbalanced meiotic products (King 1993). Rearrangements such as these, could negatively impact the fertility of a species to the point that it could reproductively isolate different population groups (Noor *et al.* 2001; Hoffman and Rieseberg 2008; Ullastres *et al.* 2014), through chromosomal speciation (Brown and O'Neill 2010, Faria and Navarro 2010). Considering this, I compared the sequence divergences (uncorrected p-

distances) of specimens, which are carriers and non-carriers of chromosome rearrangements (see Appendix: Table 2).

Within clade 1, the sequence divergence between specimens with and without inversions were low (0.3 1.1%). For example, one specimen which had inversions on four different chromosomes was almost equally divergent (0.4%) from a specimen without any chromosome rearrangements (standard karyotype), than a specimen which only had an inversion on one chromosome (0.3%). These specimens including others with novel inversions fell within the same clade and displayed similar divergence figures compared to specimens with fewer or no rearrangements. The greatest sequence divergences (≥5.5%) were between specimens with the partially bi-armed cytotype (e.g. Kroomie, Stellenbosch, Grahamstown), and specimens without (e.g. Sandile's Rest, Hogsback, Mbotyi), regardless of which rearrangements these specimens carry (see Appendix: Table 2). This is not surprising, as the two different cytotypes groups in two separate clades on the tree topology. There was however deviation from this trend. For example, two samples from Kroomie (this study), and samples from Bloemfontein and Theunissen in the Free State, were placed in clade 2 based on the sequence data, but in clade 1 based on the karyotypic data (Rambau *et al.* 2001, Engelbrecht *et al.* 2011b).

The low sequence divergence reported amongst specimens with and without inversions in clade 1 suggests that these chromosome rearrangements likely do not constitute strong reproductive isolating mechanisms. These rearrangements do not appear to influence mtDNA divergence figures (possibly since mtDNA is neutral), and thus appear to exist as floating polymorphisms (Engelbrecht *et al.* 2011b). This result corroborates the immunostaining data (Chapter 2) which showed a lack of inversion loops, suggesting that the rearrangements do not infer any deleterious consequences to the carrier, possibly due to synaptic adjustment (Coluzzi 1982, Hale 1986, Bojko 1990, King 1993, Massip *et al.* 2009, 2010). If these rearrangements

do not present negative fitness consequences to the carrier, it would explain the persistence and prevalence of these rearrangements in high frequencies. The abovementioned however remains merely speculative considering the possibility that these rearrangements drive speciation through adaptive purposes, and cannot be ruled out by the data gathered here. Regardless, these results substantiate the idea of incomplete or incipient speciation within *O. irroratus* (Engelbrecht *et al.* 2011a).

3.4.3 Phylogeographic review

The geographic distributions of the two clades are sympatric in the Alice area in the Eastern Cape. Clade 1 (*O. irroratus*) hence encompasses the Western Cape and most of the Eastern Cape whereas the range of the second lineage (*O. auratus*) extends north-eastwards from the Alice area as far north-east as Zimbabwe, also going into the interior to Gauteng and the Free State provinces. This is a region of bioclimatic transition where the fynbos biome that characterises the Western Cape meets the Grassland biome that characterises the Eastern Cape (Mucina and Rutherford 2006). Historically this region was characterised by an abundance of Afromontane forests, which became degraded and fragmented through climatic oscillations after the Last Glacial Maximum (Lawes 1990), leading to the establishment of coastal belt forests. This area, referred to as the Bedford-Gap (Lawes 1990, Lawes *et al.* 2007) presents a biogeographic break that has been shown to affect the phylogeographic patterns of a variety of mammals including *Otomys* (Engelbrecht *et al.* 2011a), *Rhabdomys* (Rambau *et al.* 2003, du Toit *et al.* 2012), *Aethomys* (Russo *et al.* 2010), *Myosorex* (Willows-Munro and Matthee 2009, 2011) and even some rodent ectoparasites of the *Polyplax* and *Listropsylla* genera (du Toit *et al.* 2013, Van der Mescht *et al.* 2015). It is thus not surprising that a similar pattern was observed for *O. irroratus* s.l.

3.4.4 Concluding remarks

In this study I demonstrated that there is shallow sequence divergence between carriers of non-carriers of chromosome inversions, and instead a high divergence between the two main cytotypes, which also corresponds to two separate mtDNA lineages and two morphometric groups which are distributed across two different climatic zones (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a). The low sequence divergence between members of clade 1 strongly suggests that the inversions (*per se*) do not constitute strong isolating barriers and likely persists as neutral polymorphisms. However, these rearrangements could potentially play a role in divergence, through adaptive purposes as the members of the respective clades occupies different bioclimatic regions. This study also demonstrated the potential of the COI gene in documenting intraspecific genetic variation in mammalian taxa, as it retrieved identical tree topologies to that of the commonly used cyt *b* gene.

Chapter 4: Concluding remarks

This study extends the sampling coverage of the species with four new localities that were analysed both cytogenetically and with mtDNA sequences. The chromosome banding data largely corroborated cytogenetic trends found in previous studies on the species (Contrafatto *et al.* 1992a, b, Rambau *et al.* 2001 Engelbrecht *et al.* 2011a). Novel findings included the range expansions of some rearrangements as well as the discovery of an inversion on OIR7 in the first cytogenetic group. The immunofluorescence data however, cast doubt on the identity the rearrangements found in this cytogenetic group. For instance, the synaptonemal complex configurations (SYCP3 data) showed no inversion loops, in addition a bi-centric chromosome was found (with CENPC antibody) in a specimen which are indicated to have inversions by G-banding data. These data then suggests that the rearrangements are not inversions, but rather centromeric shifts, with the second perceived centromere being the consequence of a recent centromere sliding event giving rise to a new epiallele (Purgato *et al.* 2015, Guilotto *et al.* 2017), which will eventually mature into the evolutionary new centromere (Piras *et al.* 2010, Rocchi *et al.* 2012). Inversions could however not be ruled out as it is possible that synaptic adjustment supressed inversion loop production (Hale 1986, Moses *et al.* 1981, Massip *et al.* 2009, 2010). Regardless, the G-banding data indicates that these rearrangements exists as floating polymorphisms, considering their presence in both heterozygous and homozygous states. It is therefore very likely that these rearrangements do not have detrimental effects on fertility, regardless of whether they exist as inversions maintained by synaptic adjustment, centromeric shifts, or a combination of the two.

The mtDNA sequence data revealed the presence of two major clades which corresponds to the two main cytogenetic groups. This data corroborated the findings of previous studies showing a cryptic lineage within *O. irroratus* with sufficient divergence in the cyt *b* gene (Bradley and Baker 2001) to be considered a separate species, *O. auratus* (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a). The sequence divergence between members of clade 1 was low, whereas the specimens of clade 2 displayed high sequence divergences of ~5% (mtDNA cyt *b*) which opens the possibility of yet another cryptic lineage to be present.

The onset of climatic oscillations which caused the contraction of habitats within the Fynbos and Grasslands biomes likely forced *O. irroratus* s.l. to retreat to suitable regions in the highlands of the Cape Fold Mountains and the Drakensberg (Taylor *et al.* 2009a). Climatic amelioration again improved conditions and opened favourable habitats allowing the species to extend its range back to lower elevations. It was in this process that clade 1, which is indicated to be younger and still actively speciating, evolved from clade 2, which conversely is older and more stable from an evolutionary perspective. This also explains the distribution patterns of the two mtDNA lineages as well as the monophyletic nature of the tree topologies, with clade 2 being basal to clade 1. As the suitable habitats of the vlei rat became isolated, chromosomal rearrangements seen in both the respective lineages got established and got fixed likely by population flush (Carson 1975, Robinson and Elder 1987). It is quite possible that the chromosomal rearrangements, specifically pericentric inversions could have facilitated clade 1 as it expanded into new suitable habitats, by harbouring genes which facilitate local adaptation in its inverted regions (Kirkpatrick and Barton 2006, Kirkpatrick 2010). The tandem fusion also probably evolved to facilitate the adaptation of vlei rat in the cold moist regions of the North East highlands going into the Drakensberg (Taylor *et al.* 1994, Brown *et al.* 1999)

Although there is strong evidence for karyotypic divergence for these two respective mtDNA groups, the division is not absolute as two specimens from Kroomie had bi-armed

chromosomes but grouped in the mtDNA clade which consisted of specimens with acrocentric karyotypes. It therefore appears that these species are still actively diverging, possibly with incomplete lineage sorting. In addition, these specimens were indistinguishable (basic field observation on phenotype) from other specimens caught in the very same locality. This again highlights the cryptic nature of the species, as no morphological divergence is evident, although molecular data clearly shows that the specimens are genetically distinct. It is thus of outmost importance to examine morphologically convergent taxa with molecular tools to prevent the underestimations of biodiversity (Gordon and Watson 1986, Lecompte *et al.* 2008, Bastos *et al.* 2011, Phukuntsi *et al.* 2016)

The animals from each of the respective main clades, although divergent enough to be considered separate species, occupy the same ecological niche. If they have however diverged on the mtDNA and chromosomal level to the extent reported here and elsewhere (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a, b), perhaps it is possible that they have diverged on other ecological levels too in terms of life history strategy, behaviour etc. Morphological divergence is clearly not completely sorted, as there were still large degrees of overlap in the cranial characters of specimens from these two groupings (Taylor *et al.* 2009a). The only form of ecological divergence which is clear between these two main clades pertains to the fact that they inhabit different biomes (Taylor *et al.* 2009a, Engelbrecht *et al.* 2011a). All of these biomes have their own unique climatic variables (temperature, rainfall etc.), vegetation compositions, fire regimes and more (Mucina and Rutherford 2006). Future studies should investigate whether there are any other ecological and biological differences between members of these two respective clades.

This study demonstrates the utility of immunofluorescence as a molecular cytogenetic tool to investigate chromosomally variable taxa in southern Africa as limited attention has been given to rodent taxa from this region. The application of this technique could provide

invaluable insight into the chromosomal evolution of mammals (Capilla *et al.* 2016). Although recombination rates could not have been addressed by this investigation, the IF data have provided some insight into neutral nature of the rearrangements reported in vlei rat. Furthermore, this study showed that the mtDNA COI gene is comparable to cyt *b* as it retrieved an identical tree topology as the cyt *b* gene, the only difference being weaker nodal support figures. Combining cyt *b* and COI sequences did not improve nodal support. Moreover, this study provided further corroborative evidence for the recognition of *O. auratus* as sister species to *O. irroratus*.

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Appendix

Table 1: List of primary and secondary antibody combinations tried. Several antibodies from different suppliers were used and alterations were made to the original protocol in attempt to successfully hybridize these antibodies. These manipulations included trials with antibodies at various concentrations, varying concentrations of the testes suspension, a range of different lispol times, fixative at low and high concentrations as well as preparation of antibodies in a different buffer agent. This table merely demonstrates effort put in to get these antibodies to work, and thus acquire recombination data.

Primary @ concentrations 1: 25, 1: 50, 1:100, 1:200 , 1: 400	**Secondary** @concentrations 1:50, 1: 100, 1:200, 1: 400, 1: 500	**Success**	**Other Manipulations**
Mouse αHuman MLH1 –BD pharmigen	Donkey αMouse FITC- Abcam	Did not work	Concentration of testes suspension 40, 60, 100, 150 and 200µl of PBS. Lipsol treatment times 1, 3, 5, 7, 10, 12, 14 and 16 minutes. 1% Paraformaldehyde fixative rather than the original 4% Antibodies prepared in 4XSSC rather than PBS/tween
	Goat αMouse FITC- Abcam	Did not work	
Mouse αHuman MLH1 –BD pharmigen αMouse MLH1- Abcam	αMouse FITC – Jacksons lab	Did not work	
	αMouse Texred- Calbiochem	Did not work	
	Donkey αMouse FITC- Abcam	Did not work	
	Goat αMouse FITC- Abcam	Did not work	
αMouse MLH1- Abcam αRabbit MLH1- Abcam	αMouse FITC – Jacksons lab	Did not work	
	αMouse Texred- Calbiochem	Did not work	
	αRabbit Cy3- Abcam	Did not work	
	αRabbit FITC-Calbiochem	Did not work	
αMouse MLH1- Santa Cruz	Donkey αMouse FITC- Abcam	Did not work	
	Goat αMouse FITC- Abcam	Did not work	
αMouse MLH1- Santa Cruz	αMouse FITC – Jacksons lab	Did not work	
	αMouse Texred- Calbiochem	Did not work	

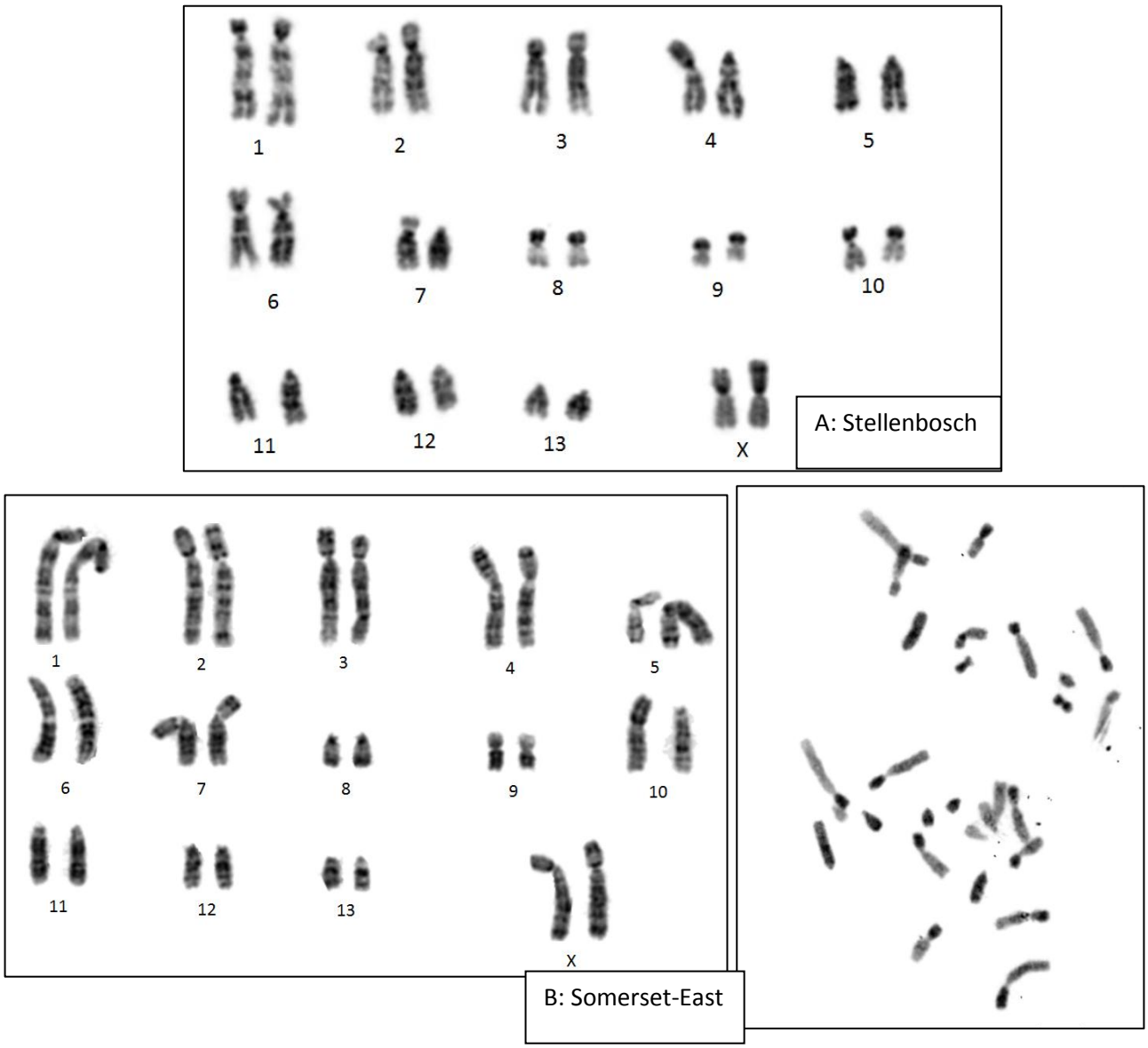
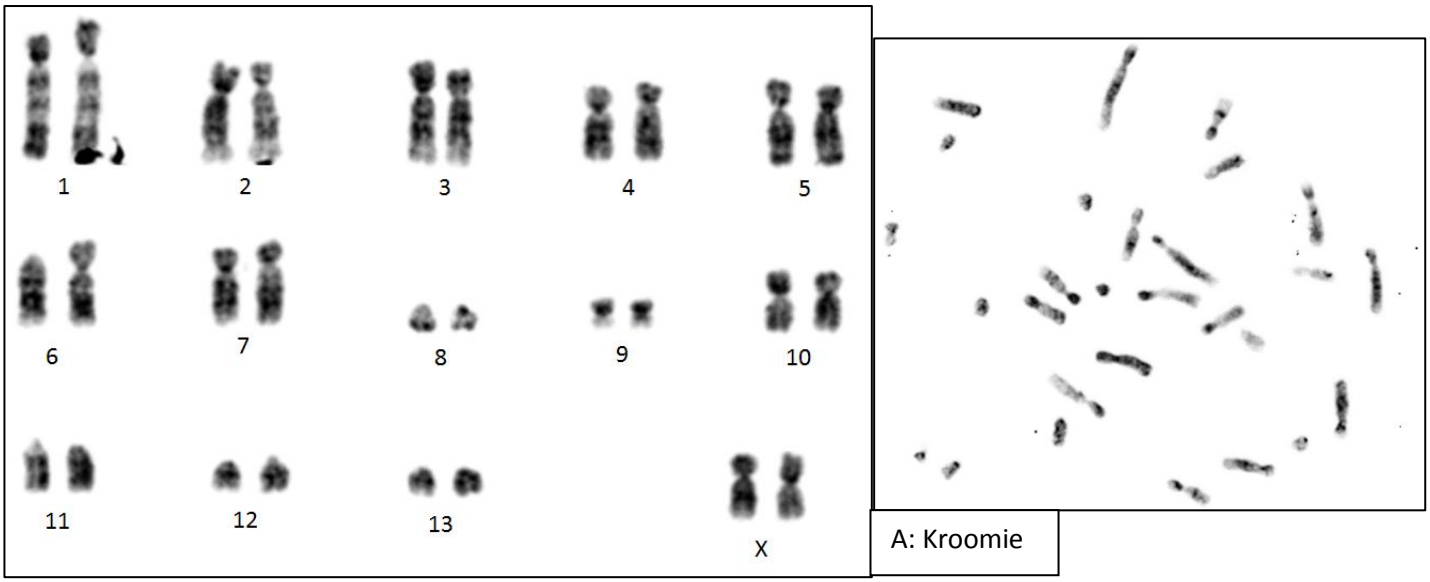
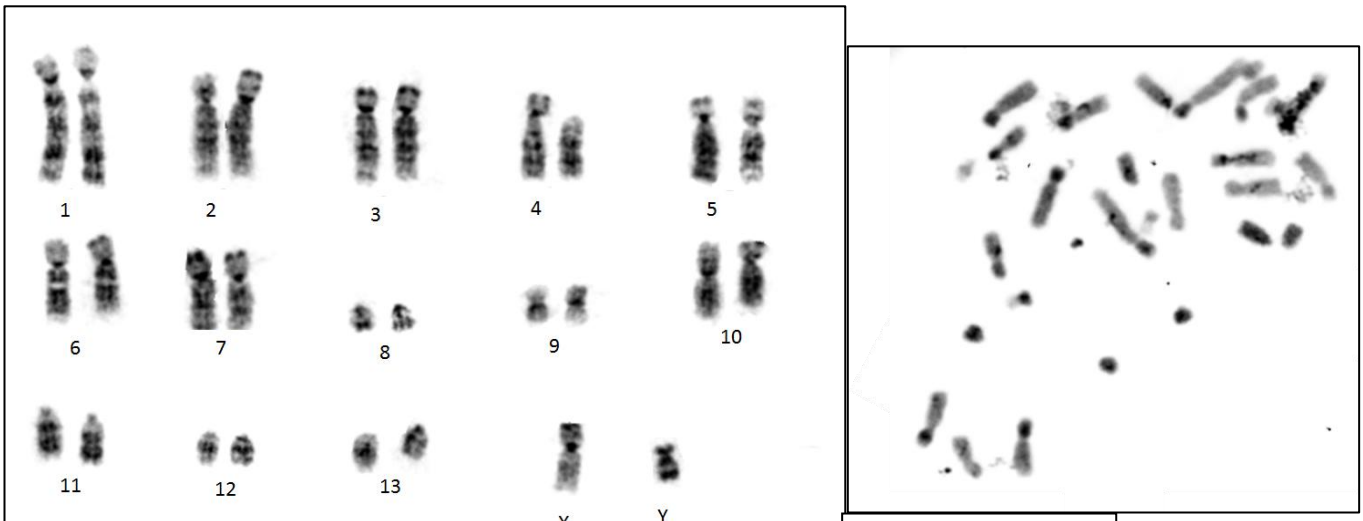


Figure 1: (A) G- banded karyotype of an individual of Stellenbosch. Three individuals were examined and all had the C cytotype configuration, with an acrocentric chromosomal pair 5. One individual was heterozygous for a new pericentric inversion on OIR 7, while the other had an inversion on OIR 6 and the last was heterozygous for the inversion on OIR10. Several specimens displayed a loss of heterochromatic short arms on OIR 4. (B) Seven specimens were analysed from Somerset-East, all with the B-cytotype. One specimen had 2 supernumerary B chromosomes increasing the 2n to 30. Three specimens had the inversion on OIR1 in heterozygous form, five specimens displayed a loss of heterochromatic short arms on OIR 4 of which one was in homozygous form, all the specimens had the inversion of OIR6 either in heterozygous or homozyogous form and lastly two specimens had the inversion on OIR 10 in heterozygous form.



A: Kroomie



B: Kenton on Sea

Figure 2: (A) Nine specimens were analysed from Kroomie, all with the B- cytotype. Two specimens had inversion on OIR1 in heterozygous form, two specimens had the inversion on OIR 6 in heterozygous form and one specimen had a newly identified inversion on OIR7 in heterozygous form. Three specimens lost heterochromatic short arms on OIR4. (B) From the four specimens analysed from Kenton on sea, three had heterozygous inversions on OIR 4 of which one also had an inversion on OIR 6. The other specimen was heterozygous for the inversion on OIR6 only. All these karyotypes had the B-cytotype.

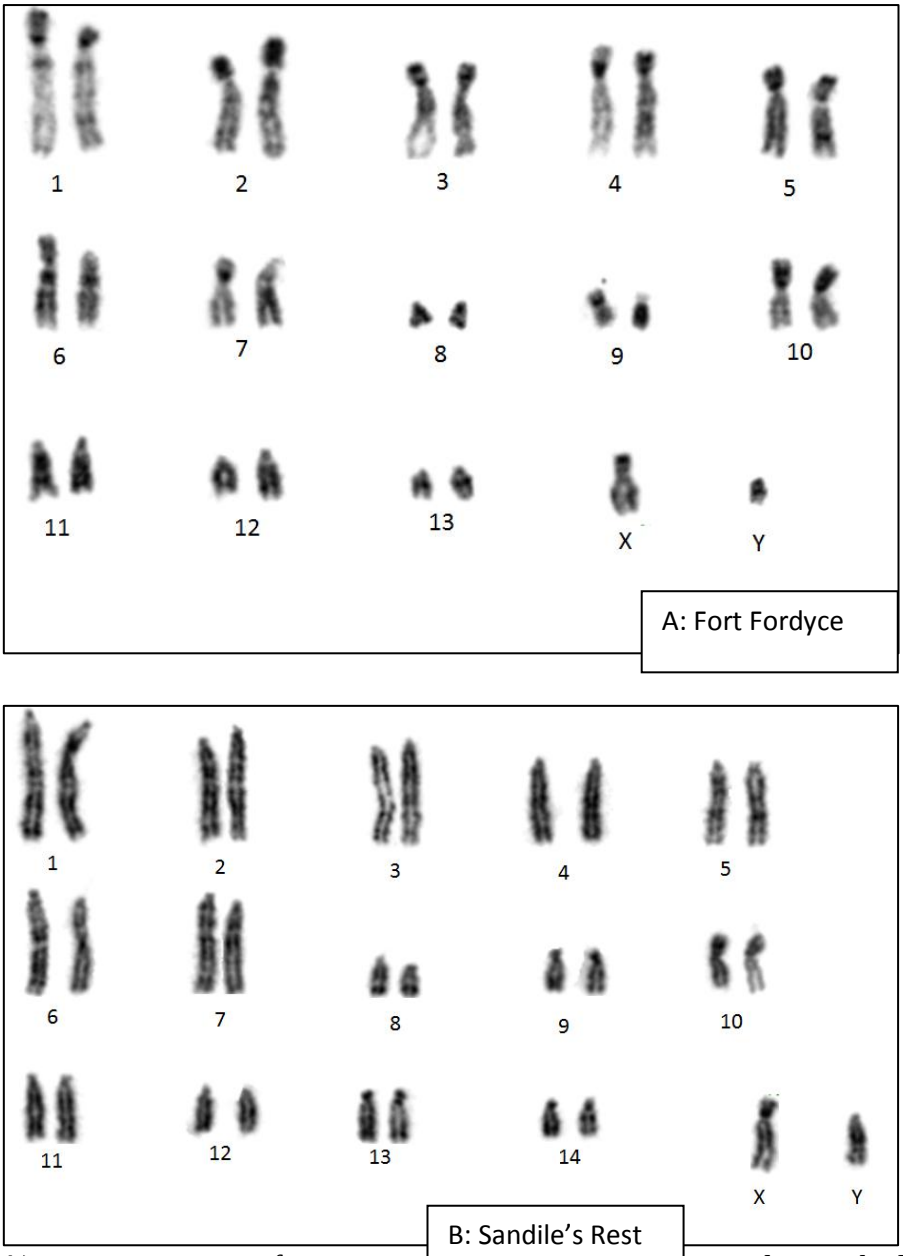


Figure 3: (A) Two specimens from Fort Fordyce had the B cytotype. One specimen was heterozygous for the inversion on OIR 1 and the other was heterozygous for the inversion on OIR6. Three specimens were analysed from Sandile's rest. These specimens displayed a 2n= 30 and had the A cytotype (A2-without compound chromosome), consisting of acrocentric chromosomes. The chromosomal pair OIR10 as well as the X was the only bi-armed chromosomes. No inversions or any other form of chromosomal rearrangements was evident in any of these three karyotypes, although it does appear that there are addition copies of the OIR9 pair.

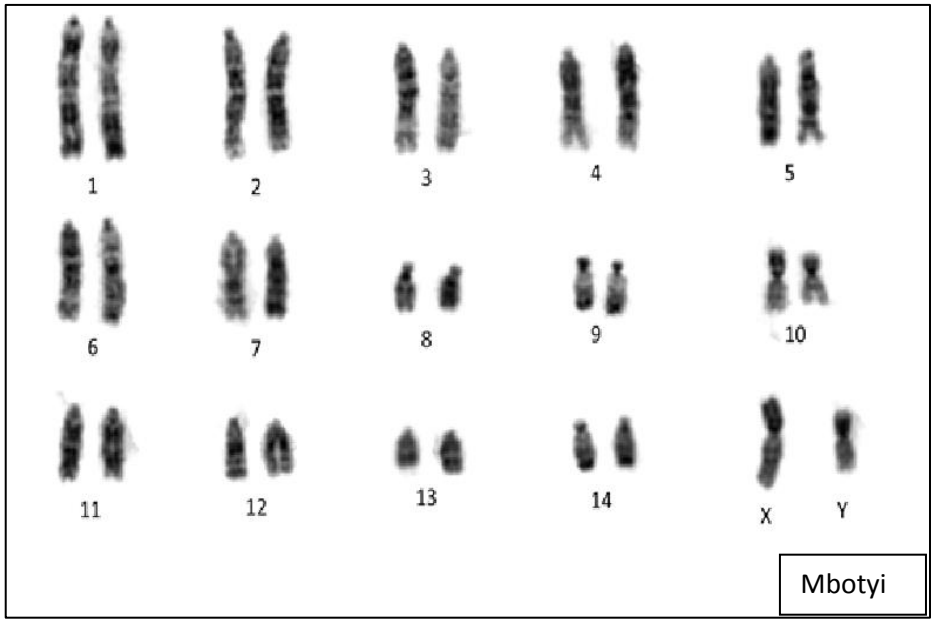


Figure 4: Two specimens from Mbotyi were karyotyped. Both specimens had the A cytotype, with acrocentric chromosomes. One specimen (above) had a 2n= 30 with the identical chromosome composition as the specimens from Sandile's Rest. The other specimen had a Robertsonian fusion between OIR1 and OIR6, and lacked certain chromosomal pairs, resulting in a 2n= 24.

Table 2: Sequence divergence values between the 62 different haplotypes retrieved with the cyt *b* gene. Values right of the 0. Decimal represents a percentage out of a hundred. Chromosome rearrangements present in the karyotypes of these specimens are listed were available.

[1] Somerset-East **2n=28 Inv 4 and 6**{Clade1}
[2] Kroomie **2n=28 No rearrangements**{Clade1}
[3] Kroomie **2n=29 No rearrangements B - Chromosome**{Clade1}
[4] Kroomie **2n=29 No rearrangements B - Chromosome** {Clade_2}
[5] Mbotyi **2n= 30 No rearrangements** {Clade_2}
[6] Mbotyi **2n=24 Rb 1/6** {Clade_2}
[7] Fort Fordyce **2n=28 inv 1,6,7**{Clade1}
[8] Sandile's Rest **2n=30 No rearrangements**{Clade_2}
[9] Kenton on Sea **2n=28 inv 4** {Clade1}
[10] Kenton on Sea **2n=28 inv 6**{Clade1}
[11] Grahamstown **2n=28 inv 1,4,6,10**{Clade1}
[12] Stellenbosch **2n=28 inv 4,7**{Clade1}
[13] Stellenbosch **2n=28 inv 6, 10**{Clade1}
[14] Stellenbosch{Clade1}
[15] Stellenbosch {Clade1}
[16] Alice{Clade1}
[17] Alice{Clade1}
[18] Sam Knott NR{Clade1}
[19] Swartberg Mts{Clade1}
[20] Bainskloof{Clade1}
[21] Algeria{Clade1}
[22] Bainskloof{Clade1}
[23] Grahamstown{Clade1}
[24] Groendal NR{Clade1}
[25] Sam Knott NR_{Clade1}
[26] Sam Knott NR {Clade1}
[27] Somerset East {Clade1}
[28] Hogsback {Clade_2}
[29] Hogsback {Clade_2}
[30] Hogsback {Clade_2}
[31] Baviaankloof {Clade1}
[32] Kroomie {Clade1}
[33] Somerset East{Clade1}
[34] Somerset East{Clade1}
[35] Grahamstown{Clade1}
[36] Beaufort-West{Clade1}
[37] Beaufort-West {Clade1}
[38] Baviaanskloof {Clade1}
[39] Gamkaskloof {Clade1}
[40] Porterville {Clade1}
[41] Kroomie {Clade1}
[42] Baviaanskloof{Clade1}
[43] Oudsthoorn {Clade1}
[44] Porterville {Clade1}
[45] Porterville {Clade1}
[46] Theunissen {Clade2}
[47] De Hoop NR {Clade1}
[48] Chingamwe Clade_2}
[49] Kamberg {Clade_2}
[50] Tygerskloof{Clade_2}
[51] Springs{Clade_2}
[52] Tygerskloof _{Clade_2}
[53] Hogsback _{Clade_2}
[54] Lemonwood Forrest{Clade_2}
[55] Karkloof_{Clade_2}
[56] Theunissen {Clade_2}
[57] Stellenbosch {Clade1}
[58] Hogsback{Clade_2}
[59] Buffelsjagsrivier{Clade1}
[60] Fort Beaufort{Clade1}
[61] Alice1{Clade1}
[62] Stellenbosch{Clade1}

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[        1     2     3     4     5     6     7     8     9    10    11    12    13    14    15    16    17    18    19    20    21    22    23
[ 1]
[ 2]  0.004
[ 3]  0.003 0.004
[ 4]  0.059 0.059 0.058
[ 5]  0.063 0.063 0.062 0.006
[ 6]  0.066 0.066 0.065 0.011 0.005
[ 7]  0.002 0.004 0.003 0.059 0.063 0.066
[ 8]  0.062 0.062 0.061 0.004 0.002 0.007 0.062
[ 9]  0.003 0.003 0.004 0.056 0.061 0.063 0.003 0.059
[10]  0.006 0.008 0.005 0.060 0.064 0.067 0.006 0.062 0.005
[11]  0.004 0.004 0.005 0.060 0.064 0.067 0.004 0.062 0.004 0.009
[12]  0.004 0.005 0.004 0.057 0.062 0.064 0.004 0.060 0.004 0.006 0.006
[13]  0.008 0.010 0.007 0.055 0.062 0.064 0.008 0.060 0.009 0.009 0.011 0.006
[14]  0.006 0.008 0.005 0.057 0.062 0.064 0.006 0.060 0.007 0.007 0.009 0.003 0.005
[15]  0.008 0.010 0.007 0.059 0.063 0.066 0.008 0.062 0.009 0.009 0.011 0.006 0.007 0.005
[16]  0.003 0.003 0.004 0.059 0.063 0.066 0.003 0.061 0.002 0.007 0.004 0.004 0.009 0.007 0.009
[17]  0.004 0.004 0.004 0.059 0.064 0.067 0.004 0.062 0.003 0.008 0.004 0.005 0.010 0.008 0.010 0.001
[18]  0.004 0.003 0.005 0.060 0.065 0.067 0.004 0.063 0.004 0.009 0.005 0.006 0.011 0.009 0.011 0.004 0.004
[19]  0.006 0.007 0.006 0.061 0.065 0.068 0.006 0.063 0.006 0.007 0.008 0.006 0.009 0.007 0.009 0.006 0.007 0.008
[20]  0.008 0.009 0.007 0.063 0.068 0.071 0.008 0.066 0.006 0.002 0.010 0.008 0.011 0.009 0.011 0.008 0.009 0.010 0.009
[21]  0.016 0.017 0.015 0.060 0.064 0.067 0.016 0.062 0.016 0.017 0.017 0.016 0.018 0.017 0.018 0.016 0.017 0.017 0.017 0.018
[22]  0.017 0.018 0.015 0.061 0.065 0.070 0.017 0.063 0.017 0.018 0.019 0.017 0.020 0.018 0.020 0.017 0.018 0.019 0.018 0.020 0.011
[23]  0.001 0.002 0.003 0.061 0.067 0.069 0.002 0.065 0.002 0.005 0.004 0.004 0.007 0.005 0.007 0.002 0.003 0.004 0.006 0.007 0.016 0.018
[24]  0.002 0.003 0.002 0.063 0.068 0.070 0.003 0.066 0.003 0.002 0.005 0.003 0.004 0.002 0.004 0.003 0.004 0.005 0.003 0.004 0.015 0.017 0.003
[25]  0.001 0.000 0.003 0.063 0.068 0.070 0.002 0.066 0.002 0.006 0.004 0.004 0.007 0.005 0.007 0.002 0.003 0.002 0.006 0.008 0.018 0.020 0.002
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[26]  0.001 0.000 0.003 0.063 0.068 0.070 0.002 0.066 0.002 0.006 0.004 0.004 0.007 0.005 0.007 0.002 0.003 0.002 0.006 0.008 0.018 0.020 0.002
[27]  0.001 0.002 0.003 0.064 0.070 0.072 0.002 0.067 0.002 0.005 0.004 0.004 0.007 0.005 0.007 0.002 0.003 0.004 0.006 0.007 0.018 0.020 0.002
[28]  0.066 0.067 0.066 0.010 0.011 0.013 0.067 0.011 0.065 0.066 0.070 0.065 0.062 0.064 0.066 0.067 0.068 0.070 0.068 0.068 0.066 0.067 0.065
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[31]  0.001 0.002 0.003 0.064 0.070 0.072 0.002 0.067 0.002 0.005 0.004 0.004 0.007 0.005 0.007 0.002 0.003 0.004 0.006 0.007 0.018 0.020 0.002
[32]  0.001 0.002 0.003 0.062 0.067 0.070 0.002 0.065 0.002 0.005 0.004 0.004 0.007 0.005 0.007 0.002 0.003 0.004 0.006 0.007 0.018 0.020 0.002
[33]  0.002 0.003 0.004 0.063 0.068 0.071 0.003 0.066 0.003 0.006 0.005 0.005 0.009 0.006 0.009 0.003 0.004 0.005 0.007 0.009 0.019 0.021 0.003
[34]  0.002 0.003 0.002 0.063 0.068 0.071 0.003 0.066 0.003 0.004 0.005 0.005 0.006 0.004 0.006 0.003 0.004 0.005 0.005 0.006 0.017 0.019 0.003
[35]  0.004 0.005 0.006 0.067 0.072 0.074 0.005 0.070 0.005 0.008 0.001 0.007 0.011 0.008 0.011 0.005 0.006 0.007 0.010 0.011 0.021 0.023 0.005
[36]  0.003 0.004 0.003 0.064 0.070 0.072 0.004 0.067 0.004 0.003 0.006 0.004 0.005 0.003 0.005 0.004 0.005 0.006 0.004 0.005 0.014 0.016 0.004
[37]  0.004 0.005 0.004 0.063 0.068 0.071 0.005 0.066 0.005 0.004 0.007 0.005 0.006 0.004 0.006 0.005 0.006 0.007 0.005 0.006 0.017 0.019 0.005
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[39]  0.006 0.007 0.006 0.066 0.071 0.073 0.007 0.069 0.007 0.006 0.010 0.007 0.008 0.006 0.008 0.007 0.008 0.010 0.001 0.008 0.019 0.021 0.007
[40]  0.006 0.007 0.006 0.061 0.068 0.071 0.007 0.066 0.007 0.006 0.010 0.005 0.002 0.004 0.006 0.007 0.009 0.010 0.007 0.009 0.019 0.021 0.007
[41]  0.002 0.003 0.004 0.065 0.070 0.072 0.003 0.068 0.003 0.007 0.005 0.005 0.009 0.006 0.009 0.003 0.004 0.005 0.007 0.010 0.019 0.021 0.003
[42]  0.006 0.007 0.006 0.065 0.071 0.073 0.007 0.068 0.007 0.006 0.010 0.007 0.009 0.006 0.009 0.007 0.009 0.010 0.007 0.009 0.017 0.019 0.007
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[44]  0.013 0.014 0.015 0.063 0.068 0.071 0.014 0.066 0.014 0.015 0.016 0.014 0.017 0.015 0.017 0.014 0.015 0.016 0.016 0.017 0.004 0.015 0.012
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[57]  0.006 0.008 0.005 0.058 0.062 0.065 0.006 0.061 0.007 0.007 0.009 0.003 0.007 0.004 0.007 0.007 0.008 0.009 0.007 0.009 0.017 0.018 0.007
[58]  0.062 0.062 0.062 0.009 0.010 0.013 0.062 0.010 0.060 0.063 0.063 0.061 0.059 0.061 0.062 0.062 0.063 0.064 0.064 0.067 0.061 0.062 0.067
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[60]  0.004 0.004 0.004 0.059 0.063 0.066 0.004 0.062 0.003 0.008 0.004 0.005 0.010 0.008 0.010 0.003 0.004 0.004 0.007 0.009 0.017 0.018 0.002
[61]  0.003 0.003 0.004 0.058 0.062 0.065 0.003 0.061 0.002 0.007 0.004 0.004 0.009 0.007 0.009 0.002 0.003 0.004 0.006 0.008 0.016 0.017 0.002
[62]  0.008 0.010 0.007 0.058 0.062 0.065 0.008 0.061 0.009 0.009 0.011 0.004 0.009 0.005 0.009 0.009 0.010 0.011 0.009 0.011 0.015 0.017 0.010

[       24    25    26    27    28    29    30    31    32    33    34    35    36    37    38    39    40    41    42    43    44    45    46
[24]
[25]  0.003
[26]  0.003 0.000
[27]  0.003 0.002 0.002
[28]  0.066 0.067 0.067 0.067
[29]  0.065 0.066 0.066 0.066 0.010
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[34]  0.002 0.003 0.003 0.003 0.066 0.065 0.061 0.003 0.003 0.004
[35]  0.006 0.005 0.005 0.005 0.071 0.069 0.065 0.005 0.005 0.006 0.006
[36]  0.001 0.004 0.004 0.004 0.067 0.066 0.062 0.004 0.004 0.005 0.003 0.007
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[37]  0.002 0.005 0.005 0.005 0.066 0.065 0.060 0.005 0.005 0.006 0.004 0.009 0.003
[38]  0.004 0.005 0.005 0.005 0.066 0.065 0.061 0.005 0.005 0.006 0.004 0.009 0.005 0.006
[39]  0.004 0.007 0.007 0.007 0.070 0.068 0.064 0.007 0.007 0.009 0.006 0.011 0.005 0.006 0.009
[40]  0.004 0.007 0.007 0.007 0.064 0.065 0.059 0.007 0.007 0.009 0.006 0.011 0.005 0.006 0.009 0.009
[41]  0.004 0.003 0.003 0.003 0.068 0.067 0.063 0.003 0.003 0.004 0.004 0.006 0.005 0.006 0.006 0.009 0.009
[42]  0.004 0.007 0.007 0.007 0.068 0.067 0.065 0.007 0.007 0.009 0.006 0.011 0.003 0.006 0.009 0.009 0.009 0.009
[43]  0.005 0.009 0.009 0.009 0.063 0.064 0.058 0.009 0.009 0.010 0.007 0.012 0.006 0.005 0.010 0.010 0.003 0.010 0.010
[44]  0.013 0.014 0.014 0.014 0.066 0.065 0.061 0.014 0.014 0.015 0.015 0.017 0.012 0.015 0.017 0.017 0.017 0.015 0.015 0.016
[45]  0.014 0.015 0.015 0.015 0.067 0.066 0.062 0.015 0.015 0.016 0.016 0.018 0.013 0.016 0.018 0.018 0.018 0.016 0.016 0.017 0.001
[46]  0.073 0.071 0.071 0.070 0.044 0.047 0.044 0.072 0.072 0.071 0.073 0.075 0.072 0.072 0.073 0.076 0.071 0.072 0.072 0.070 0.064 0.065
[47]  0.003 0.007 0.007 0.007 0.068 0.065 0.061 0.007 0.007 0.008 0.006 0.010 0.005 0.003 0.007 0.008 0.006 0.008 0.006 0.006 0.016 0.017 0.071
[48]  0.061 0.062 0.062 0.063 0.041 0.044 0.042 0.063 0.063 0.064 0.062 0.064 0.063 0.061 0.062 0.065 0.060 0.064 0.060 0.059 0.064 0.065 0.029
[49]  0.069 0.068 0.068 0.068 0.012 0.009 0.026 0.068 0.066 0.067 0.070 0.071 0.071 0.070 0.070 0.072 0.067 0.069 0.072 0.066 0.067 0.068 0.045
[50]  0.070 0.069 0.069 0.067 0.044 0.047 0.044 0.070 0.070 0.068 0.071 0.072 0.072 0.070 0.071 0.073 0.068 0.070 0.072 0.067 0.064 0.065 0.004
[51]  0.071 0.070 0.070 0.068 0.043 0.046 0.043 0.071 0.071 0.070 0.072 0.073 0.071 0.071 0.072 0.074 0.070 0.071 0.071 0.068 0.063 0.064 0.001
[52]  0.071 0.070 0.070 0.068 0.047 0.050 0.045 0.071 0.071 0.070 0.072 0.073 0.071 0.071 0.072 0.074 0.070 0.071 0.071 0.068 0.063 0.064 0.003
[53]  0.065 0.066 0.066 0.066 0.005 0.013 0.024 0.066 0.064 0.065 0.065 0.069 0.066 0.065 0.065 0.068 0.063 0.067 0.067 0.062 0.065 0.066 0.047
[54]  0.067 0.068 0.068 0.068 0.012 0.006 0.026 0.068 0.066 0.067 0.067 0.071 0.068 0.067 0.067 0.070 0.067 0.069 0.070 0.066 0.067 0.068 0.049
[55]  0.071 0.070 0.070 0.068 0.043 0.046 0.043 0.071 0.071 0.070 0.072 0.073 0.071 0.071 0.072 0.074 0.070 0.072 0.071 0.068 0.063 0.064 0.003
[56]  0.072 0.071 0.071 0.068 0.045 0.046 0.041 0.071 0.071 0.070 0.072 0.074 0.071 0.071 0.072 0.075 0.070 0.072 0.071 0.068 0.063 0.064 0.003
[57]  0.004 0.007 0.007 0.007 0.066 0.065 0.059 0.007 0.007 0.009 0.006 0.011 0.005 0.006 0.009 0.008 0.006 0.009 0.009 0.007 0.017 0.018 0.070
[58]  0.068 0.068 0.068 0.070 0.006 0.014 0.025 0.070 0.067 0.068 0.068 0.072 0.067 0.068 0.068 0.071 0.066 0.070 0.068 0.065 0.066 0.067 0.044
[59]  0.004 0.007 0.007 0.007 0.064 0.065 0.059 0.007 0.007 0.009 0.006 0.011 0.005 0.006 0.009 0.008 0.002 0.009 0.009 0.003 0.017 0.018 0.070
[60]  0.003 0.002 0.002 0.002 0.067 0.066 0.062 0.002 0.002 0.003 0.003 0.005 0.004 0.005 0.005 0.007 0.007 0.003 0.007 0.009 0.014 0.015 0.069
[61]  0.003 0.002 0.002 0.002 0.067 0.066 0.062 0.002 0.002 0.003 0.003 0.005 0.004 0.005 0.005 0.007 0.007 0.001 0.007 0.009 0.014 0.015 0.071
[62]  0.006 0.009 0.009 0.010 0.066 0.065 0.059 0.010 0.010 0.011 0.009 0.013 0.005 0.009 0.011 0.011 0.009 0.011 0.009 0.010 0.015 0.016 0.068
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[	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
[47]																
[48]	0.059															
[49]	0.069	0.039														
[50]	0.069	0.031	0.044													
[51]	0.070	0.030	0.044	0.003												
[52]	0.070	0.031	0.047	0.006	0.004											
[53]	0.065	0.041	0.011	0.047	0.046	0.048										
[54]	0.066	0.043	0.004	0.049	0.048	0.052	0.011									
[55]	0.070	0.030	0.043	0.005	0.002	0.006	0.046	0.048								
[56]	0.070	0.030	0.046	0.007	0.004	0.004	0.048	0.050	0.006							
[57]	0.007	0.059	0.064	0.067	0.068	0.068	0.065	0.067	0.069	0.070						
[58]	0.069	0.039	0.011	0.044	0.044	0.047	0.007	0.012	0.042	0.045	0.062					
[59]	0.007	0.057	0.061	0.067	0.068	0.068	0.063	0.067	0.069	0.070	0.007	0.059				
[60]	0.007	0.061	0.063	0.065	0.066	0.066	0.066	0.068	0.068	0.068	0.008	0.062	0.008			
[61]	0.007	0.060	0.062	0.068	0.069	0.069	0.066	0.068	0.070	0.071	0.007	0.062	0.007	0.003		
[62]	0.009	0.059	0.064	0.067	0.066	0.066	0.065	0.067	0.067	0.067	0.002	0.060	0.009	0.010	0.009	